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THE INFLUENCE OF HORMONES ON ENZYMES

BY

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THE INFLUENCE OF HORMONES ON ENZYMES*

Conference Chairmen: R. I. DORFMAN AND E. D. GOLDSMITH†

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* This series of papers is the result of a Conference on *The Influence of Hormones on Enzymes*, held by the Section of Biology of the New York Academy of Sciences, June 5 and 6, 1951.

† Acknowledgment is hereby made to R. I. Dorfman and E. D. Goldsmith for their collaboration with the Editor in the preparation of this monograph of the Annals.

INTRODUCTION

E. D. Goldsmith and Ralph I. Dorfman

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Doctor Edwin Cohn expressed it well when he said "the study of enzymes may be characterized as the oldest of the arts and the newest of the professions." Fermentation has been known and utilized by man for many centuries, but enzymology did not come of age until Buchner demonstrated in 1895 that fermentation could be catalyzed by a factor isolated from yeast and Sumner crystallized the enzyme urease from the jack bean 25 years ago.

Since the pioneer discovery of the hormone, secretin, by Bayliss and Starling in the early years of this century, the knowledge of the role played by the internal secretions in the regulation of animal growth and functions has increased tremendously. With these advances in our knowledge in endocrinology and enzymology has come the realization that the hormones may be producing their effects by influencing certain enzymes or enzyme systems. This influence may be exerted: (1) by means of changes in tissue enzyme concentrations; (2) by the hormone functioning as a component of an enzyme system; or (3) by direct or indirect effect on accelerators and/or inhibitors of enzyme systems.

The task at hand is to accumulate experimental data to prove or disprove these hypotheses. To that end, a beginning has been made, but a great deal remains to be done. This monograph brings together in one volume the representative data in the field and the diverse ideas of interested investigators. It is hoped that, as a result of this volume, the available data will be critically evaluated, correlated, and integrated so that additional productive research in this field will follow.

We are most grateful to the contributors both for their presentations at the Conference and for their manuscripts. We are grateful also to Doctors G. Pincus, W. C. Stadie, and A. White, who presided over the three sessions at the Conference.

RECENT STUDIES ON THE *IN VIVO* AND *IN VITRO* EFFECT OF HORMONES ON ENZYMES

By Charles D. Kochakian*

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The material for this paper will be confined to unpublished and some recently published data concerned with (1) further correlations of changes in arginase activity with hormone-induced protein anabolism and catabolism and (2) the *in vivo* and *in vitro* effects of testosterone on tissue respiration.

Changes in Arginase Activity in Relation to Changes in Protein Metabolism

Protein Anabolism; Androgens. The many previous studies[1, 2] have amply demonstrated that the arginase activity of the liver is not altered by the administration of androgens but that that of the kidney is greatly increased. In the present studies, the arginase activity of the kidney and liver was determined during various phases of the effect of androgen on body weight and urinary nitrogen in the rat[3] (see FIGURE 1). When testosterone propionate was administered at 1.0 mg. per day to the castrated rat,[4] there was a gradual but small increase in the size of the kidney. The arginase activity of this tissue progressively and greatly increased during the protein anabolic phase, and continued to increase throughout the period in which the retention of nitrogen was disappearing and even after nitrogen equilibrium had been restored. Thus, there does not seem to be any direct correlation between the arginase activity of the kidney and the retention of nitrogen from dietary sources. The increase in arginase activity might well be concerned with other processes in the body. It is now known[5] that, even though androgen-induced nitrogen retention disappears, other protein anabolic and catabolic reactions are occurring. This can be seen, for example, in the continued increase in size of both the kidney and seminal vesicles and prostates.

The arginase activity of the liver in these animals was not influenced by the administration of the androgen.

Results similar to the above were obtained in castrated rats fed *ad libitum* and in hypophysectomized rats under metabolic study. Thus, in the latter animals, the greatly decreased arginase activity of the kidney was restored but that of the liver was not affected.

Protein Catabolism; Diabetes.[6] The two types of diabetes, phlorizin and alloxan, provide further conditions under which arginase activity might be correlated with changes in protein metabolism. Furthermore, the studies were carried out at several concentrations of protein in the diet and by superimposing the protein anabolic effect of testosterone propionate on the protein catabolic effect of the diabetes. The rats in these studies were on the metabolic regimen.[3]

Phlorizin Diabetes. The phlorizin in experiments 1 and 2 (TABLE 1)

* The studies reported in this paper were carried out in the Department of Physiology and Vital Economics, School of Medicine and Dentistry, University of Rochester and, except where noted, were supported by grants from the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

caused a decrease in body weight of 8 and 16 grams respectively, which was partly prevented by the androgen whether the injections were begun simultaneously with the phlorizin (experiment 2) or 7 days later (experiment 1).

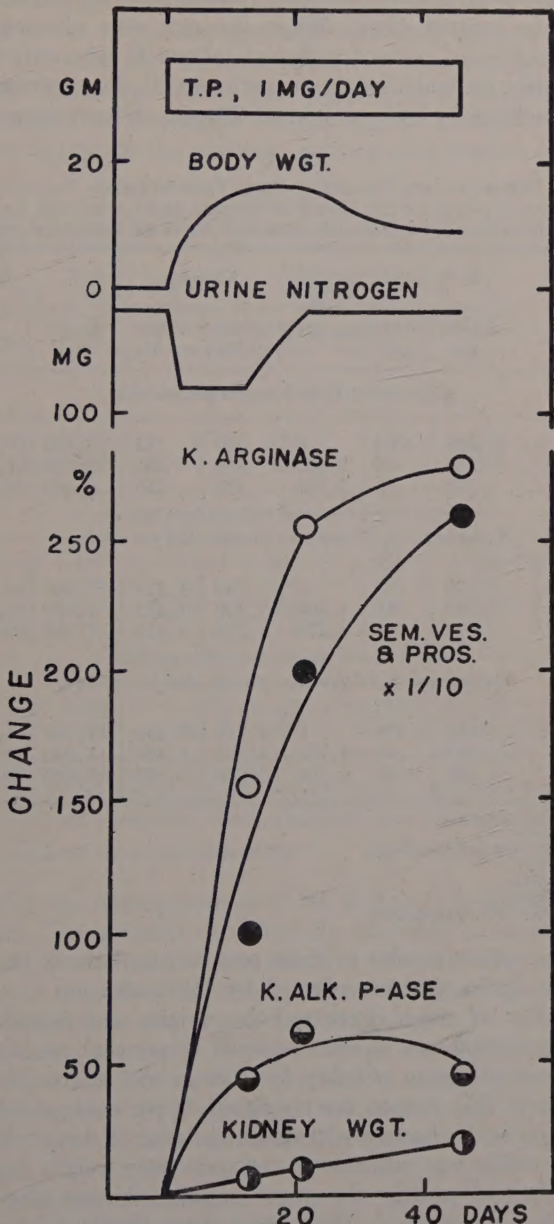

FIGURE 1. A comparison of the protein anabolic effect of testosterone propionate with tissue enzyme changes. The rats were fed an amount of food which would maintain caloric and nitrogen equilibrium. (Graph prepared from data of KOCHAKIAN, C. D. & E. ROBERTSON. Arch. Biochem. **29:** 114, 1950.)

At the same time, the androgen produced a decrease in the nitrogen excretion equal to the increase stimulated by the phlorizin but did not influence the glucosuria. It should be noted that the extra protein catabolism stimulated by the phlorizin is 20 and 15 per cent, respectively, for the rats on the 30 and 70 per cent protein diets. When the rats were allowed to increase their food intake in an amount sufficient (about 35 per cent) to offset the effect of phlorizin on body weight (experiment 3), the androgen produced a maximum increase in body weight of 20 gm. and nitrogen retention of

TABLE 1

THE EFFECT OF PHLORIZIN AND PHLORIZIN PLUS TESTOSTERONE PROPIONATE (T.P.) ON THE ARGINASE ACTIVITY OF THE LIVER OF NORMAL AND CASTRATED RATS ON DIETS OF DIFFERENT PROTEIN CONTENT (5 RATS IN EACH GROUP)

Treatment	*Body weight*		*Glucose urine*	*Nitrogen*		*Liver*	
	final gm.	*change* gm.	mg./day	*intake* mg./day	*urine* mg./day	*weight* mg.	*arginase U/gm.*
Experiment 1, 18 per cent protein diet							
Normal	283	+10	0	320	243	9,850	10,740 ± 810[a]
Phlorizin[b]	268	−8	2,860	320	291	8,560	11,890 ± 620
Phlorizin + T.P.[c]	278	−5	2,550	320	250	8,660	10,520 ± 1,080
Experiment 2, 70 per cent protein diet for 19 days							
Castrate	320	−3	0	1,230	1,114	7,660	16,730 ± 1,020
Phlorizin[d]	307	−16	3,400	1,230	1,285	7,650	15,620 ± 1,330
Phlorizin + T.P.[e]	315	−8	3,470	1,230	1,118	7,560	19,090 ± 1,310
Experiment 3, 70 per cent protein diet for 94 days							
Castrate	431	+4	0	1,310	1,136	11,540	14,840 ± 800
Phlorizin[f]	365	+6	4,770	1,775	1,589	11,030	29,400 ± 1,300
Phlorizin + T.P.[g]	400	+20	4,760	1,786	1,528	13,670	23,900 ± 1,400

[a] Standard error of the mean.
[b] 30 mg./day, 12 days.
[c] 1.0 mg./day, 5 days preceding autopsy.
[d] 60 mg./day, 5 days.
[e] 0.5 mg./day, 5 days.
[f] 60 mg./day, 94 days.
[g] 1.0 mg./day, 13 days preceding autopsy.

61 mg. per day, values similar to those seen in non-diabetic castrated rats.[3] The glucosuria again was not affected by the androgen.

Phlorizin, after 12 days, decreased the weight and protein content of the liver of the normal rats on the restricted 18 per cent protein diet. The androgen at one milligram per day for 5 days was unable to compensate for the decrease. The rats on the restricted 70 per cent protein diet, however, showed no such changes with phlorizin after 5 days. Furthermore, when the food intake was sufficient to maintain body weight (experiment 3), the liver weight and protein also were maintained even after 94 days of phlorizin at 60 mg. per day. The androgen produced an increase in both weight and protein content of the liver of these rats.

The arginase activity of the liver was increased[7,8] by the 70 per cent protein diet within 19 days, and an extension of the experiment to 94 days produced a further increase in total activity but not in concentration. The phlorizin did not alter the activity of this enzyme in the experiments with either the 18 or the 70 per cent protein diets until the rats on the 70 per cent protein diet were allowed to increase their food intake to compensate for the energy lost in the urine. The increase in the enzyme activity was of special interest, for it occurred only when the cobaltous ion was used in the determination of the arginase activity and was no longer evident

TABLE 2

EFFECT OF ALLOXAN DIABETES ON THE ARGINASE ACTIVITY OF THE LIVER

Diabetes days	*No. rats*	*Final body wt. gm.*	*Glucose urine mg./day*	*Nitrogen*		*Liver*	
				intake[a] mg./ day	*urine mg./day*	*weight mg.*	*arginase U/gm.*
				18 per cent protein diet			
Normal	5	283	0	336	243	9,850	10,740 ± 810
145	3	191[b]	8,670	516	479	9,330	11,330 ± 200
				30 per cent protein diet for 30 to 44 days			
Castrate	5	319	0	458	365	6,850	10,340 ± 760
30	2	349	22	552	404	9,540	12,620 ± 1,300
30–54	3	343	4,330	815	625	10,110	11,700 ± 610
				70 per cent protein diet for 19 days[c]			
Normal	1	380	0	1,430	1,232	11,830	18,280
380	1	244[d]	1,640	1,400	1,310	10,930	20,960

[a] Food intake adjusted to maintain body weight.
[b] Emaciated, no body fat, average loss in body weight 65 gm. Cataracts in eyes. Blood glucose at autopsy 390, 447, and 172 mg.
[c] Preceded by 18 per cent protein diet. Nitrogen intake 555, urine nitrogen 378, and urine glucose 5,450 mg./day.
[d] Emaciated, no body fat, loss in body weight 81 gm.

after storage of the homogenate at 5° for 9 days. It seems then that the higher protein diet directly or indirectly provided a co-activator under the stimulus of phlorizin which was effective with the cobaltous but not the manganous ion, and that this substance was destroyed by autolysis. The presence of more than one arginase is suggested.

The testosterone propionate did not change the arginase activity under any of the above conditions. The decrease in experiment 3 is only a reflection of the increase in liver weight without a concomitant increase in arginase activity. The total amounts of arginase in both groups of rats were identical.

Phlorizin produced an increase in kidney weight and protein which were further increased by the androgen. The effect of protein content was questionable in the experiments of shorter duration, but was quite marked

in the experiments of longer duration, when the androgen prevented the edema produced by the phlorizin. The arginase activity of the kidney of the rats on the 18 per cent protein diet was decreased by phlorizin and restored by the androgen. On the other hand, the rats on the 70 per cent protein diet exhibited an increase in arginase activity in proportion to the increase in kidney weight. The androgen produced a further increase which was, as expected, more evident, +233 per cent, in the rats treated with the androgen for the longer period, 13 days.

Alloxan Diabetes. The alloxan produced the classical symptoms of diabetes, including cataracts of the eyes and emaciation (TABLE 2). The rats gained in weight at first, due to a greater ingestion of food, but then steadily lost weight, due primarily to a loss in body fat. The protein loss was never

TABLE 3

EFFECT OF TESTOSTERONE PROPIONATE (T.P.) ON THE ARGINASE ACTIVITY OF THE LIVER OF ALLOXAN DIABETIC (150 DAYS) CASTRATED RATS

Treatment	*No. rats*	*Final body wt. gm.*	*Glucose urine mg./day*	*Nitrogen*		*Liver*	
				intake[a] *mg./day*	*urine mg./day*	*weight mg.*	*arginase U/gm.*
30 per cent protein diet for 145 days							
Control......	5	363	5,880	759	643	11,060	15,370 ± 1,380
T.P.[c]........	5	377	8,080	880	775	12,300	13,670 ± 250
70 per cent protein diet for 19 days[b]							
Control......	4	343	3,350	1,630	1,570	12,170,	25,740 ± 900
T.P.[c]........	4	346	3,690	1,745	1,634	12,620	27,000 ± 1,400

[a] Food intake adjusted to maintain body weight.
[b] Preceded by 140 days on the 30 per cent protein diet.
[c] T.P. in oil solution injected at 0.5 mg./day for 5 days.

very great. Indeed, the liver and kidney increased in size and protein content, while the accessory sex organs decreased to as much as 20 per cent of normal. The degree of glucosuria, as expected, decreased on replacement of the carbohydrate of the diet by protein.

The arginase activity of the liver was not changed by the diabetes whether the rats showed a mild or a severe diabetes or whether they were on the 18 or the 30 per cent protein diet. One diabetic rat was of special interest, for, although its body weight had been greatly decreased as a result of 380 days of intense diabetes, its liver weight and arginase activity showed the same degree of increase under the stimulus of the 70 per cent protein diet as that of a normal rat of the same age.

The injection of testosterone propionate in alloxan diabetic rats produced the expected effects on nitrogen excretion, body weight, and accessory sex organs. The prolonged (145 days) ingestion of the 30 per cent protein diet by the diabetic rats (TABLE 3) resulted in an increase of the arginase activity (*cf.* TABLE 2), which, however, was not so great as that produced by the

70 per cent protein diet for the much shorter period, 19 days. This may be an indirect effect due to stimulation of the adrenal cortex. The androgen was unable to alter these changes in arginase activity of the liver.

The kidneys of all the diabetic rats showed varying degrees of sclerosis, which was related to the severity of the diabetes. Furthermore, the kidneys increased in size with a concomitant increase in nitrogen content until sclerosis set in, then there was a further increase in kidney size but not of nitrogen content. The androgen was able to produce the usual increases in the size, the protein content, and the arginase activities of the kidneys in spite of the varying degrees of sclerosis.

Protein Catabolism; Glucocorticoids. In the adrenalectomized[9] and normal[10] male rat, no change in the liver arginase activity is produced by glucocorticoids in spite of the stimulation of intense protein catabolism accompanied by glyconeogenesis. On the other hand, an increase in the arginase activity of the liver of rats has been noted[11] after the administration of glucocorticoids for several days. The following studies[12] were carried out in castrated mice of approximately 4 months of age in order to elucidate further the relationship of liver arginase activity to cortisone-induced protein metabolic changes.

The subcutaneous implantation of an approximately 14 mg. pellet of cortisone acetate produced a sharp increase in body weight accompanied by a marked increase in urinary nitrogen excretion and a decrease in the nitrogen balance from approximately +20 mg. per day to zero (FIGURE 2). After 7 days, the decrease in body weight and the increase in nitrogen excretion ceased and an immediate and marked increase in food intake with a restoration of the nitrogen balance to its original level occurred. These changes in protein metabolism were accompanied by a rapid decrease in lymphatic tissue during the protein catabolic phase (FIGURE 3), as exemplified by the complete disappearance of the thymus by the 7th day and the decrease of the spleen to roughly 30 per cent of that of its original size where it was maintained on further treatment. The kidney, on the other hand, began to increase slightly in size between the 2nd and 7th days, and the liver showed an initial increase after two days of administration of the cortisone acetate but then gradually decreased so that about 20 per cent of its weight had been lost by 30 days.

The arginase activity of the kidney both in units per gram and in total units increased immediately and rapidly after administration of cortisone acetate and reached a peak at 7 days or shortly thereafter. The arginase activity of the liver, on the other hand, showed only a small and questionable increase after two days of administration of cortisone acetate and did not show a great increase until the initial protein catabolic phase had disappeared. Then the liver arginase activity was considerably increased on both a unit per gram and a total unit basis and was maintained throughout the period of further stimulation by the hormone. Identical differences in enzyme activity were obtained when the enzyme was determined by first preheating the homogenate with manganous chloride at 50° for one hour. Furthermore, in the experiment of 21 days duration, the homogen-

ates were dialyzed at 5° against 0.01 molar sodium barbital for 24 hours, then the arginase activities were compared with undialyzed samples at

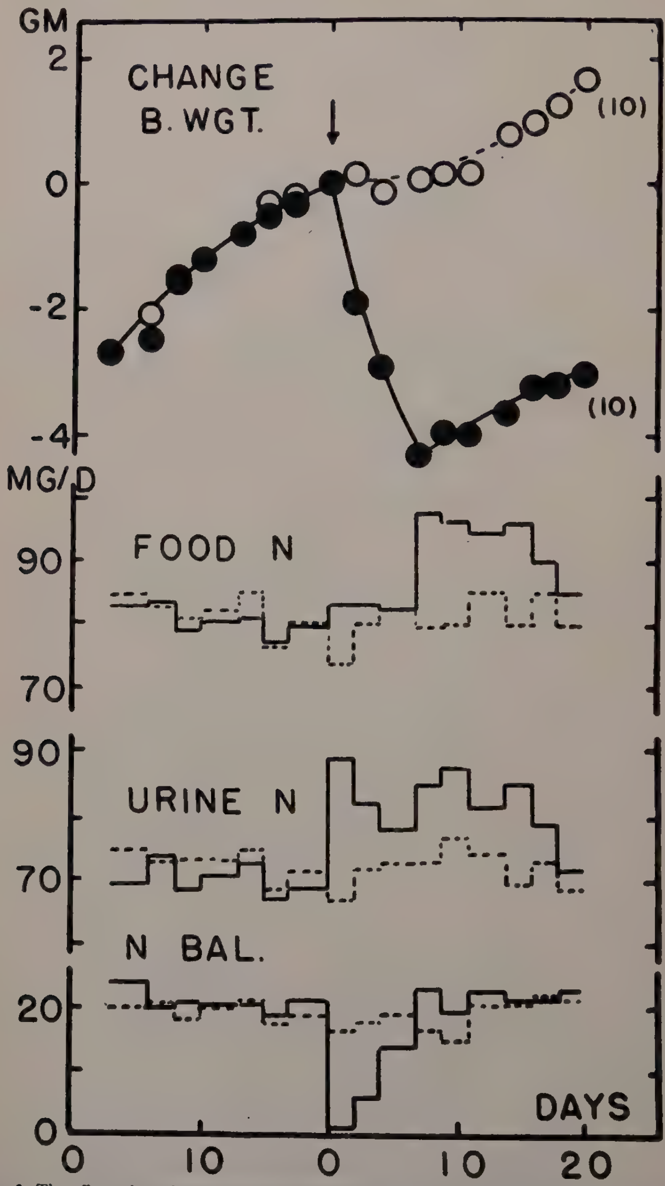

FIGURE 2. The effect of cortisone acetate on castrated mice. The cortisone acetate was implanted subcutaneously as a 13–15 mg. pellet at the time indicated by the arrow. The values for the control mice are indicated by the broken line and those of the cortisone treated mice by the solid lines (ten mice in each group). Average body weights at time of pellet implantation were: controls, 31.0 ± 0.9; cortisone acetate, 30.8 ± 1.0 gm. Many of the body weight values during the pre-treatment period were identical for the control and experimental groups. Therefore, these values for the control group are masked in the graph. Identical results were obtained in 3 other series of experiments (reprinted from KOCHAKIAN, C. D. & E. ROBERTSON. 1951. J. Biol. Chem. 190: 481).

pH 7.50, 8.65, and 9.45 at the regular and ten-fold concentrations of $CoCl_2$ and $MnCl_2$. The effect of the cortisone acetate was not altered under any of these conditions. The activity of the enzyme over this wide range of

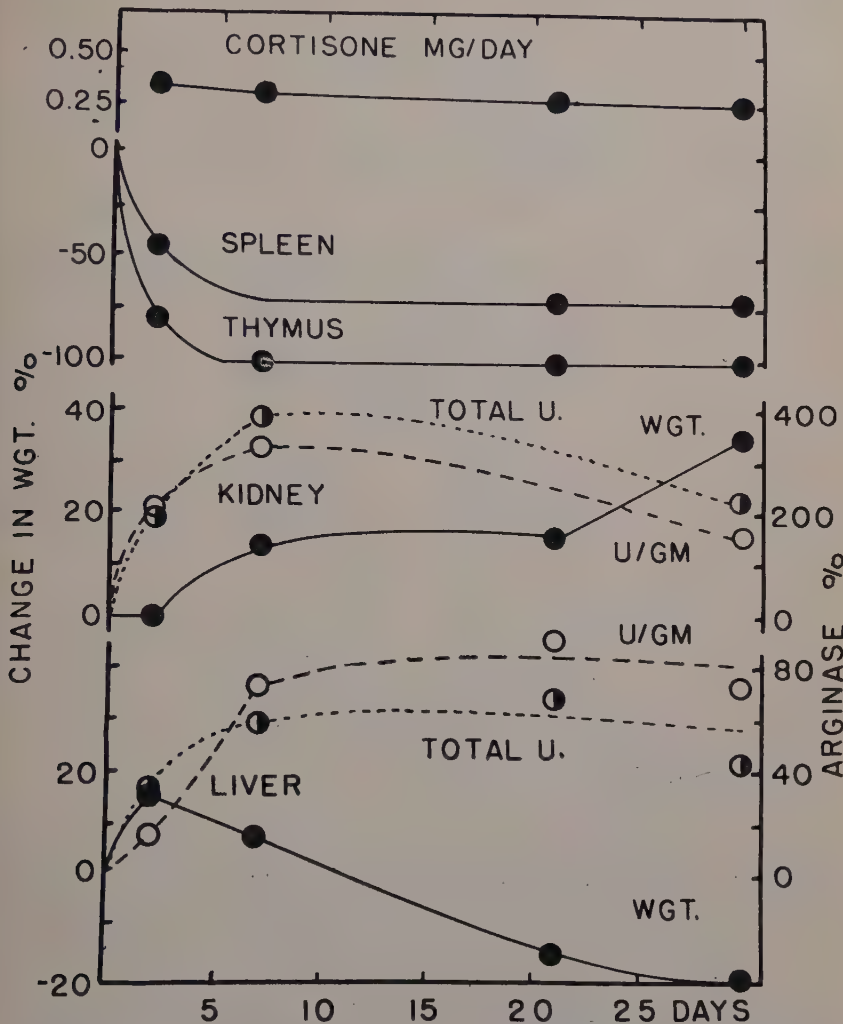

FIGURE 3. A comparison of the effect of cortisone acetate on arginase and organ weights of the castrated mouse. Identical changes in arginase activity were obtained when $MnCl_2$ was substituted for $CoCl_2$ in the arginase determination (reprinted from KOCHAKIAN, C. D. & E. ROBERTSON. 1951. J. Biol. Chem. **190:** 481).

pH is of interest. The arginase activities also were determined after storage of the tissues for 2, 7, 9, and 18 days at 5°C. without any changes in the degree of increase produced by the cortisone acetate, even though the absolute values had decreased.

Discussion. The marked increase in kidney arginase activity suggests

that glucocorticoids and androgens influence a common intermediary metabolic process. The increase in both kidney weight and enzyme activity stimulated by the cortisone acetate, however, never attained the maximum induced by androgens. Furthermore, the increase in arginase activity after cortisone administration occurred very rapidly and before the kidney had increased in size. The delayed appearance of the increase in arginase activity of the liver indicates that this enzyme is not associated with the initial intense protein catabolism which seems to be primarily involved in a mobilization of the labile lymphatic tissues. The increase might be a reflection of several possible changes. Alterations in amounts or nature of metabolic products by the cortisone acetate could change the amount of

TABLE 4

EFFECT OF CASTRATION AND TESTOSTERONE PROPIONATE (T.P.) ON TISSUE RESPIRATION

	No. mice	Body wt. gm.	T.P. absorbed mg./56 days	Kidney		Liver Q_{O_2}
				mg.	Q_{O_2}	
Normal male mice						
Control	11	21.6		373	17.7 ± 1.48[b]	6.1 ± 0.75[c]
Vitamin C[a]	10	20.1		395	19.2 ± 1.15	7.6 ± 0.64
T.P.[d]	12	18.7	7.6	444	16.9 ± 0.46	6.4 ± 0.51
T.P. + Vit. C	10	19.2	7.9	479	15.4 ± 1.05	5.7 ± 0.84
Castrated[e] male mice						
Control	11	22.9		291	14.2 ± 1.14[b]	5.7 ± 0.61[c]
Vitamin C	9	20.6		283	16.4 ± 0.95	5.4 ± 0.48
T.P.	10	19.5	6.6	497	16.0 ± 0.78	5.5 ± 0.60
T.P. + Vit. C	11	18.3	7.7	420	17.2 ± 0.70	5.5 ± 0.64

[a] 20 mg./100 gm. body wgt./day injected subcutaneously for six days before autopsy.
[b] R.Q. 0.75 ± 0.03; 0.67 ± 0.01.
[c] R.Q. 0.58 ± 0.04; 0.71 ± 0.05.
[d] Pellet (20 ± mg.) of T.P. implanted subcutaneously at time of castration; autopsy 56 (42–70) days later.
[e] Castration at 12–17 gm.

activators and inhibitors or stimulate the production of more of the enzyme. The maintenance of the effect of the cortisone acetate after dialysis of the homogenates would indicate that low molecular weight activators were not produced.

Tissue Respiration Studies

In Vivo.[13] The absorption of testosterone propionate from a 20 mg. pellet implanted subcutaneously in Buffalo-Marsh mice at the time of castration (12 to 17 gm.) produced, after 56 (42–70) days, the expected changes in kidney mass but no significant changes in Q_{O_2} (TABLE 4). The amount of respiration per total mass of kidney, therefore, is decreased by castration and increased by the androgen in proportion to the changes of tissue mass. The small decrease as the result of castration is not substantiated by statistical analysis but still may be real. These changes are similar to those observed for d-amino acid oxidase.[14]

The oxygen consumption of the liver also is not affected by either castration or treatment with the androgen alone or in combination with vitamin C.

It was of interest that the respiratory quotient of the kidney was de-

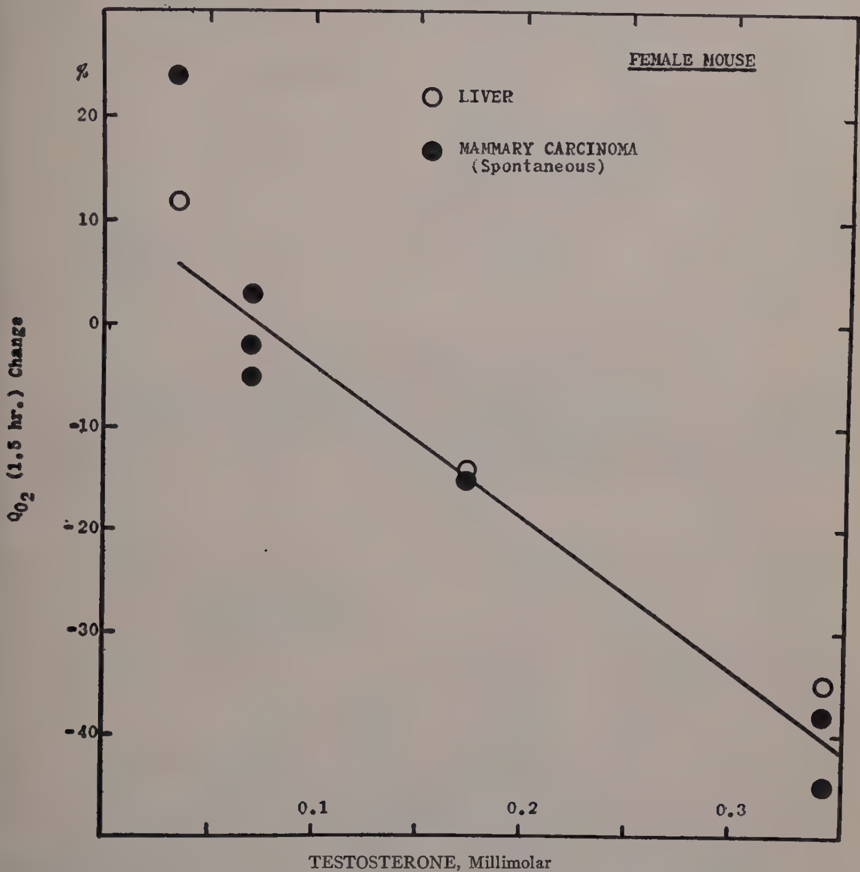

FIGURE 4. The effect of testosterone on the respiration of liver and spontaneous mammary carcinoma slices of female mice. The Warburg method was used. The two tissues were removed from the same animal. The atmosphere was air, and the measurements of oxygen uptake were made at 30-minute intervals for 1.5 hours. The medium (*cf.* ref. 18) was Ringer's solution buffered with M/150 phosphate. The testosterone was dissolved in alcohol and added to the medium to give a 264 millimolar solution. The control flasks contained an identical concentration of alcohol. Each determination was carried out in duplicate or triplicate.

creased from 0.75 ± 0.03 to 0.67 ± 0.01 by castration and restored to normal by the administration of androgen. The R.Q. of the liver, on the other hand, was increased from 0.58 ± 0.04 to 0.71 ± 0.05 by castration and restored to normal by the androgen administration.

In Vitro. The addition of testosterone by solution in alcohol so that the final buffer medium of 3 ml. was 264 millimolar in alcohol produced an initial slight increase (*cf.* ref. 6) followed by a decrease in the respiration of liver and spontaneous mammary carcinoma obtained from either dba or C_3H mice, (FIGURE 4). The decrease in respiration was proportional to

the amount of testosterone added and did not occur until the concentration of testosterone exceeded 0.075 millimolar. On the other hand, slices of

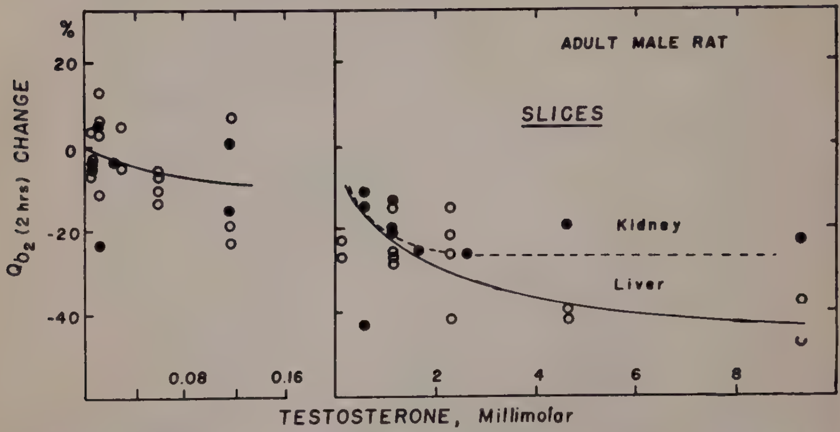

FIGURE 5. The effect of testosterone on the respiration of liver and kidney slices of adult male rats. Warburg method used. Atmosphere was oxygen. The slices were prepared with the Stadie-Riggs microtome. Kreb's or Ringer's phosphate medium used. Testosterone dissolved in alcohol and an equal amount added to the control flasks. Final concentration varied from 28 to 560 millimolar, depending on amount of testosterone added.

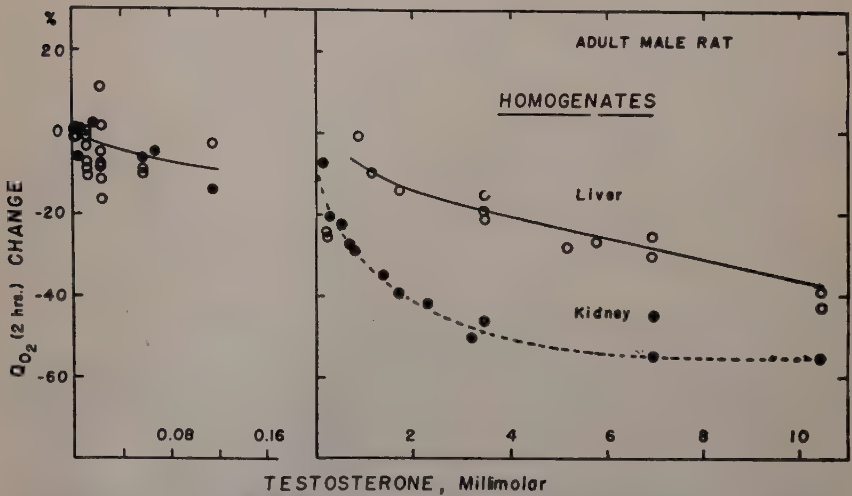

FIGURE 6. The effect of testosterone on the respiration of the homogenates of the liver and kidney of the adult male rat. Testosterone was homogenized with the tissue in either Kreb's or Ringer's phosphate solution. Warburg technic used with an atmosphere of oxygen. Measurements made at 20-minute intervals for the first hour and at 30-minute intervals for the second hour.

primary Brown-Pearce epithelioma incubated with 0.28 millimolar testosterone (3 flasks) showed no change in respiration from controls (4 flasks).

The above studies were extended to the liver and kidney of the adult ale rat.[15] The testosterone again produced a rapid decrease in the res-

piration of both the liver and kidney. At the "very high" concentrations of testosterone, the liver slices seemed to be more sensitive than the kidney slices (FIGURE 5). No evidence for an increase in oxygen respiration similar to that observed in the mouse[16] could be detected for either of the tissues, in spite of many studies at very low concentrations of testosterone. The respiration rate of homogenates (FIGURE 6) prepared from these tissues also was rapidly decreased by the addition of testosterone. In this instance, the kidney definitely was affected to a greater degree than the liver.

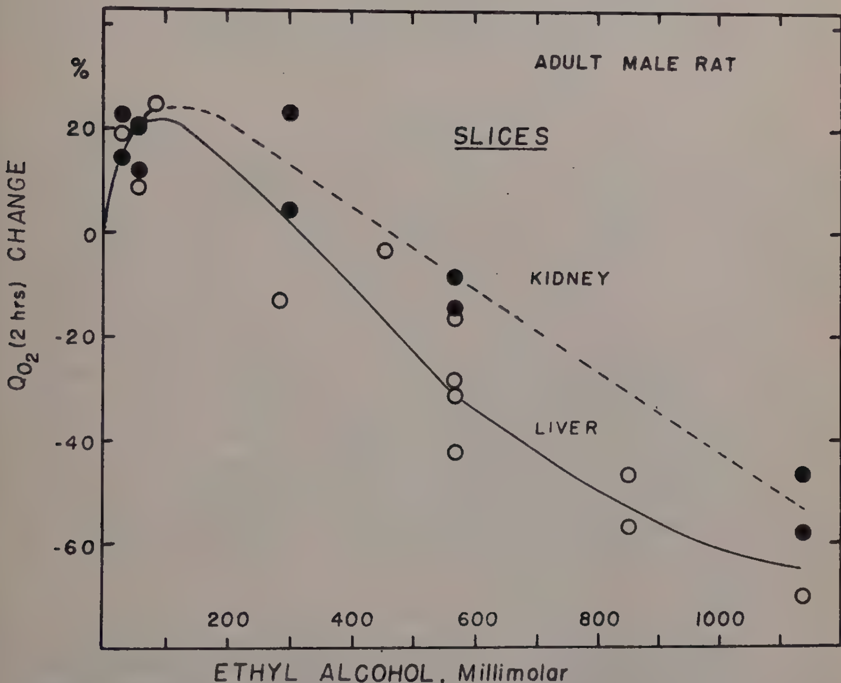

FIGURE 7. The effect of ethyl alcohol on the respiration of liver and kidney slices of the adult male rat. Warburg technic used with an atmosphere of oxygen.

Here again, no evidence for stimulation of respiration was obtained by the use of extremely small amounts of testosterone.

Since, in the experiments with tissue slices, the testosterone was added to the buffer medium by solution in small amounts of alcohol, it seemed worth while to repeat[17] the effect of alcohol on tissue respiration. The ethyl alcohol (FIGURE 7) at first increased the oxygen consumption of both liver and kidney slices. This effect then wore off and a period of gradually increasing inhibition of oxygen consumption occurred. The liver seems to be affected sooner than the kidney by the alcohol. In both instances, however, the decrease in respiration did not occur until very great amounts of alcohol were added as compared to the relatively small amounts of testosterone.

Discussion. It is to be noted that the inhibition of respiration occurs only after the concentration of testosterone in the fluid medium has greatly exceeded the physiological level as indicated by the renotrophic and androgenic effect in the mouse[1] and the protein anabolic effects in the rat.[3] The inhibitory effect of testosterone, moreover, was not evident in the tissues of the intact mouse when administered in excessive amounts for a prolonged period. Indeed, the respiration of the kidney increased in proportion to the increase in kidney mass.

The failure of testosterone to inhibit the respiration of primary Brown-Pearce epithelioma indicates that this tumor either lacks the particular enzyme system or metabolizes the testosterone in a different manner.

Summary

Testosterone propionate produced an increase in arginase activity of the kidney of rats which continued even after the protein anabolic effect had "worn off." This effect was evident in castrated rats and in phlorizin and alloxan diabetic rats maintained on diets containing 18, 30, or 70 per cent protein (casein). The arginase activity of the liver was not affected by the diabetes or the androgen. The high protein diet on prolonged administration produced identical increases in the liver arginase activity of the normal and diabetic rats. The increases were enhanced with increase in amount of protein and duration of feeding. Cortisone acetate produced an immediate and prolonged increase in the arginase activity of the kidney of mice, but the increase in the liver arginase activity was not evident until the initial intense protein catabolism had disappeared.

The administration of testosterone by a subcutaneously implanted pellet produced an increase in kidney respiration of normal and castrated mice in proportion to the increase in kidney weight. The respiration of the liver was not affected.

The addition of testosterone in amounts greater than calculated physiological needs decreased the respiration of slices of liver and spontaneous mammary carcinoma of mice and slices and homogenates of liver and kidney of adult male rats. The respiration of primary Brown-Pearce epithelioma was not changed.

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β-GLUCURONIDASE AND THE ACTION OF STEROID HORMONES

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It may be desirable at the outset to define the goals of research in the field of enzymes and the steroid hormones. In the first place, it is understandable that the explanation of the biological action of the sex steroids should be sought in the realm of enzymatic phenomena. Thus, as we know, the generalization that most biochemical reactions in the living organism are catalyzed by specific enzymes is widely held and accepted. Moreover, the success which has been realized in establishing the participation of various vitamins in essential specific enzyme systems of the cell has, in turn, led to the expectation that the steroid hormones may similarly be shown to be components of important enzyme systems.

General Considerations. First, some of the concepts will be outlined which may apply to the participation of steroids in tissue enzyme systems. Reference will be made only to the estrogenic hormones presented in FIGURES 1 and 2.

FIGURE 1 is a simplified picture of estrogen metabolism which indicates the main pathways of estrogen in the body. As can be seen, two forms of estrogen occur in the body, free and conjugated. The free estrogen may either be excreted mostly in the bile or be degraded oxidatively in the liver by non-specific oxidases. The conjugate of estrogen (either its glucuronide or sulfate) is excreted in the urine. Both free estrogen and its conjugates will stimulate growth processes in the secondary sex tissues. Accordingly, it is felt that the explanation of the action of the estrogens in terms of enzyme phenomena should be sought in these mechanisms labeled A and B, which may possibly be one and the same process.

The utilization of estrogen as an integral component of an enzyme system is most simply represented in FIGURE 2. Enzyme X, which is the term signifying that the enzyme is hypothetical and as yet unidentified, should be the limiting factor in tissue growth stimulated by estrogen. If we may reason by analogy from experiences in the enzyme-vitamin field, all vitamin coenzymes which have been studied thus far are acidic in nature. It may not be too unreasonable to predict that, should the sex steroids be found to constitute coenzymes, these hormones will possess acid functions.

Estrogen may participate in an enzyme system as a specific substrate, as in FIGURE 2. Perhaps the enzyme-substrate complex has a physiological function in growth which is distinct from its enzymological significance.

It is necessary in a field as new and as confused as that constituting steroid-enzyme phenomena to distinguish between primary and secondary enzyme reactions resulting from hormonal action. A primary enzyme reaction of hormone-induced growth, in this regard, may be defined as one in which the steroid participates directly, either as coenzyme, substrate, activator, or inhibitor. Accordingly, secondary enzyme reactions of hor-

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mone-induced growth are defined as those which are seen in growing tissue in which the steroid hormone is not a component of the enzyme system. It would be reasonable to expect the primary reactions to be associated with processes of nuclear division, whereas the secondary reactions may be concerned with the formation of new cytoplasm. These latter considerations are admittedly pure speculation.

Correlation of β-Glucuronidase Activity and Estrogen Action. It is now intended to review some of the more important facts which relate the behavior of an enzyme, β-glucuronidase, to the action of the estrogenic hormones. β-Glucuronidase is an enzyme, widely distributed in mammalian tissues, which catalyzes the hydrolysis of many β-glucuronides, *e.g.*, menthol and phenolphthalein glucuronides. These two reactions have been

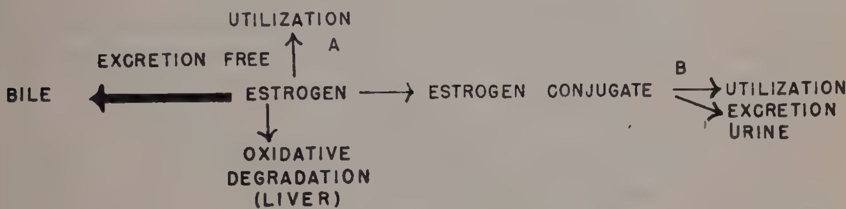

FIGURE 1.

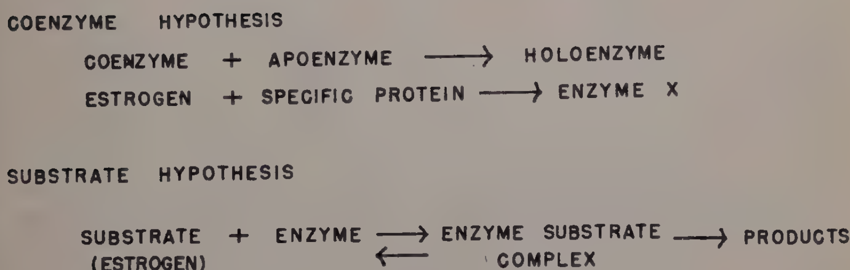

FIGURE 2.

utilized as the basis of methods of assay for β-glucuronidase activity. In the hydrolysis, the aldehyde group of glucuronic acid is liberated, and a measurement can then be made of the increase in reducing power of the β-glucuronidase digest employing standard conditions. One unit of β-glucuronidase here is defined as one milligram of glucuronic acid liberated per hour from one gram of tissue.[1, 7] In the second reaction, use is made of the characteristic red color given by phenolphthalein in alkali which is not given by phenolphthalein glucuronide.[2, 3] Accordingly, one unit of β-glucuronidase acting on the substrate, phenolphthalein glucuronide, is defined as one microgram of phenolphthalein liberated per hour under standard conditions per gram of tissue. The optimum pH of the enzyme varies between pH 4.5 and 5.2, depending on the substrate employed.[4] The dissociation constant of the enzyme-substrate complex, the so-called Michaelis constant, Km, has been determined for a number of substrates (TABLE 1), and it may be seen that the enzyme has a relatively strong

affinity for estriol glucuronide. In addition, the enzyme hydrolyzes the glucuronides of pregnandiol and pregnanetriol and of urinary corticoids. So it is clear that β-glucuronidase is a tissue protein which does have an affinity for certain steroid glucuronides.

Our investigations of the nature of this enzyme, due mainly to the efforts of Dr. Bernfeld, have shown that β-glucuronidase does contain a coenzyme which is still unidentified. The action of this coenzyme, however, may be duplicated by adding desoxyribonucleic acid to the system.

The evidence which indicates a relationship between the behavior of β-glucuronidase and the estrogenic hormones was first published from the author's laboratory in 1944.[6] Previously, in 1939,[1] it was demonstrated that the administration of glucuronidogenic drugs, such as menthol and borneol, to mice and dogs resulted in an elevation in β-glucuronidase activity of the liver, kidney, and spleen and not in the ovary, uterus, or testis. Inasmuch as these animals were conjugating the terpene alcohols with glucuronic acid, and since, by definition and in a number of instances by direct

TABLE 1
MICHAELIS CONSTANTS AND ENZYME-SUBSTRATE AFFINITIES

β-Glucuronidase hydrolysis of the glucuronide of	*Km.*	*1/Km.*
Estriol[4]	0.0005	2000
Borneol[4]	0.01	100
Menthol[4]	0.004	250
Phenol[22]	0.0035	286
Phenolphthalein[2]	0.00005	20,000

proof, we know that enzymes catalyze reversible reactions, it seemed reasonable to suggest that β-glucuronidase was indeed catalyzing the synthesis of β-glucuronides in tissue. The increase in enzymatic activity which was observed in this experiment was interpreted as reflecting a process of "adaptation" of the enzyme to substrate. There are many examples in the literature of the so-called "adaptive enzymes," which are produced by microorganisms in response to a specific substrate in the medium.

The development of this hypothesis of adaptive response of the synthetic activity of β-glucuronidase to substrate, although it may very well be completely wrong, has proven very useful in the design of our experiments on estrogens.

(a) *Animal Experiments.* The main observations in these experiments are the following: Upon ovariectomy, a marked decrease occurs in uterine β-glucuronidase of the female mouse.[6] When estrogens, both naturally-occurring and synthetic,[1, 7] were administered to castrate female mice in physiological amounts, there followed an increase in β-glucuronidase in the uterus accompanied by no significant change in activity in the liver, kidney, and spleen. Testosterone propionate given in amounts of 100 micrograms over three days did not increase uterine β-glucuronidase to the extent seen with only two micrograms of estradiol benzoate, nor did it, in larger amounts,

antagonize the β-glucuronidase response of the estrogen-stimulated uterus.[7] The suggestion was made, therefore, that the function of the enzyme in the uterus was to conjugate estrogen with glucuronic acid as a step in the utilization of the steroid glucuronide by the tissue.

The following studies have been completed in human subjects. Human endometrium is the tissue richest in β-glucuronidase, and it undergoes changes during the menstrual cycle. Moreover, it is known (following the work of Cohen and Marrian) that, as pregnancy proceeds, there is a more or less progressive increase in the urine of the glucuronides of the estrogens and the metabolites of progesterone, such as pregnandiol and pregnanolone. Immediately after parturition, the amounts of these urinary steroid glucuronides diminish markedly. Observations made independently by the author[8] and by MacDonald and Odell[9] have shown that the serum β-glucuronidase becomes elevated during pregnancy. Bernard and Odell,[10] in 1950, reported increased liver and blood serum β-glucuronidase in pregnant rats. Moreover, the post-partum fall in serum β-glucuronidase activity can be partially prevented by the administration of stilbestrol in amounts of 5 mg./day.[11] We have shown, recently, that the serum β-glucuronidase can be elevated about 100 per cent above control levels in post-menopausal women treated with as little as 5 mg./stilbestrol per day for seven days.[12] Cohen and Huseby[13] reported that patients with far-advanced cancer of the breast similarly increase their serum β-glucuronidase in response to estrogen therapy. It would appear likely, therefore, that β-glucuronidase of serum under certain conditions in humans reflects estrogen activity. Is this a phenomenon specific to estrogens? The answer is "no" on the basis of our own observations. Methylandrostenediol, which is a weak androgen with nitrogen anabolic properties, will, in a small percentage of cases, produce an elevation in serum β-glucuronidase.

Behavior of β-Glucuronidase in the Liver and Kidney. A description will now be given in some detail, of observations which have been made on the β-glucuronidase activity in the non-sex organs. The reason, in part, for studying the non-sex organs was the difficulty of explaining the rather marked elevation in serum β-glucuronidase in estrogen-treated subjects as reflecting activity of the secondary sex tissues alone.

One will recall that, in our earlier experiments with castrate mice, the administration of estrogen in amounts as large as 72 γ over three days of injection did not cause any alteration in the liver, kidney, and spleen β-glucuronidase. While Harris and Cohen[14] have confirmed this observation, Kerr and coworkers,[15] in Edinburgh, claimed that the injection of estrone but not of estriol or estradiol produced an increase in liver β-glucuronidase.

A new observation on the factors which control the level of β-glucuronidase in tissue was made by Morrow and his associates.[16] They reported that inbred mice of the C_3H strain which have a high susceptibility for mammary cancer exhibited extremely low liver β-glucuronidase activity. Moreover, levels of the enzyme in kidney and spleen were reduced, although not to the same extent.

It was felt, therefore, that the C_3H mice would provide suitable experi-

mental animals in which the possibility would be increased of observing significant alterations in the β-glucuronidase of the non-target organs. The results of our experiments with stilbestrol are given in TABLE 2. Groups of five to eight animals of the strain designated were given 2.5 milligrams of stilbestrol over a period of five days, each daily dose being injected subcutaneously in a volume of 0.1 cc. peanut oil. At the end of this time, the animals were sacrificed, the livers and kidneys pooled separately, and β-glucuronidase determinations were performed on aqueous homogenates of the fresh tissues. Control animals received injections of peanut oil. It

TABLE 2
STILBESTROL EXPERIMENTS (2.5 MG./5 DAYS)

Sex	Strain	*β-Glucuronidase activity (units/gram)*			
		Liver		*Kidney*	
		control	*experimental*	*control*	*experimental*
♂	White Swiss	3363	5350	2015	1465
♂	C_3H	284	1012	434	380
♂	C_3H	284	1780	434	428
♀	C_3H	323	980	256	236

TABLE 3
EXPERIMENTS WITH TESTOSTERONE PROPIONATE (5 MG./5 DAYS)

Sex	Strain	*β-Glucuronidase activity (units/gram)*			
		Liver		*Kidney*	
		control	*experimental*	*control*	*experimental*
♂	C_3H	284	421	434	1540
♀	C_3H	323	327	256	866

should be kept in mind that an unphysiologically excessive amount of estrogen is being administered.

While an increase of perhaps 60 per cent in the liver glucuronidase might have occurred in the white Swiss mice, the alterations observed in the C_3H mice were much more marked, (*i.e.*, of the order of 200 to 400 per cent). It is to be noted, first, that the highest liver β-glucuronidase activity observed in the stilbestrol-treated C_3H mice was still much below the level observed in livers of white Swiss mice and, second, that the kidney β-glucuronidase was not affected by stilbestrol administration.

The next experiment which suggested itself was to adminster androgen in place of estrogen to C_3H mice. We found a surprising result (TABLE 3), namely, that following testosterone administration the liver β-glucuronidase showed no alteration, but the kidney enzyme increased very significantly. The same effects were seen with methylandrostenediol, a steroid to which we had reference before.

It was, of interest, therefore, to determine the ability of the kidney to increase its β-glucuronidase as a function of duration of hormone action.

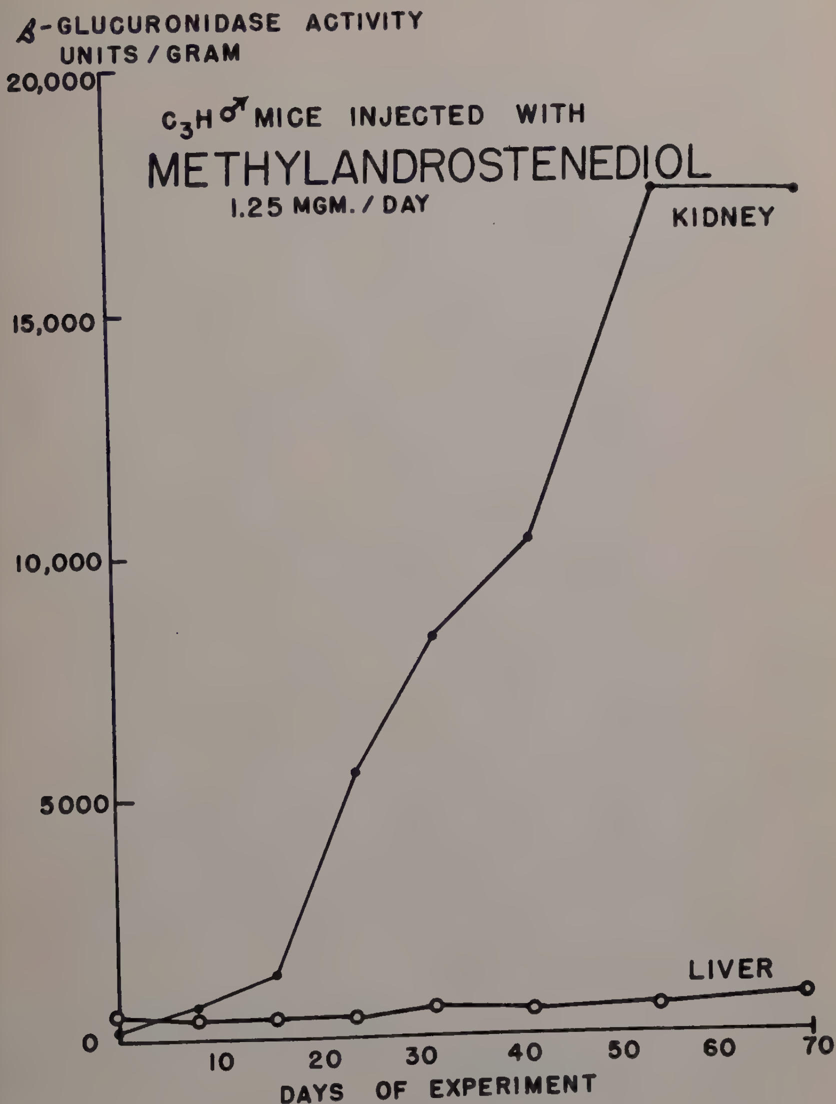

FIGURE 3.

Accordingly, intact adult male C_3H mice were injected with 2.5 mg. of methylandrostenediol in 0.1 ml. of aqueous suspension every other day for 70 days. At regular intervals during the experiment, three mice were sacrificed and the livers and kidneys were removed, pooled separately, and their β-glucuronidase activities determined. These activities are related with time in FIGURE 3. It is evident that the kidney β-glucuronidase in-

creases progressively with time to a level 50 times that observed in the controls. Throughout the course of this experiment, no significant change in the liver enzyme was seen. Castrate male or female mice receiving androgen show the same phenomenon. Similar experiments with white Swiss male mice showed a 10-fold increase in kidney β-glucuronidase. Studies of the same type on intact female C_3H mice are now in progress.

Although our investigations of the effects of steroid hormones on non-sex organs in C_3H mice are not complete, the following facts have been established: Stilbestrol given to C_3H mice of either sex stimulates liver β-glucuronidase and not the enzyme of the kidney. This liver effect is barely detectable in white Swiss mice. On the other hand, androgen produces a striking increase in the kidney β-glucuronidase in C_3H mice without any noticeable change in the activity of the liver enzyme. These experiments were performed with unphysiological amounts of hormones. It may be stated, then, that these studies indicate not only that genetic factors seem important in controlling the level of β-glucuronidase in the body tissues, as Morrell first stated, but also that these genetic factors may determine the quantitative ability of the β-glucuronidase of liver and kidney to respond to estrogen and androgen.

It may be possible, in the future, to examine androgens with respect to their relative ability to stimulate kidney β-glucuronidase activity in C_3H mice. It will be interesting to determine whether this response is related to the protein-anabolic or to the androgenic properties of the compounds to be tested. Preliminary studies, in collaboration with Dr. Homburger, are now under way utilizing measurements of renotrophic, clitoritrophic, and the kidney epithelium protective properties of certain steroids in mice with one ligated ureter.

Role of β-Glucuronidase in Vivo. The purpose of the next part of this presentation is to review the present status of our ideas on the role of β-glucuronidase in the mammalian organism. It should be emphasized at the beginning that there are only two chemical reactions which β-glucuronidase may conceivably catalyze: one, the hydrolysis of glucuronides to form the aglucurone and glucuronic acid; and the other, the synthesis of glucuronides from a mixture of the aglucurone and glucuronic acid. Interpretations of changes in tissue of the activity of this enzyme must be referred back to these two possibilities.

Kerr[17] and Levvy,[18] in Edinburgh, have observed elevated β-glucuronidase activity associated with processes of cellular repair in livers of mice receiving menthol and other toxic substances and claim to have seen abnormally high β-glucuronidase activity in states of rapid cellular growth, as in regenerating liver and in the organs of infant mice. Accordingly, they have interpreted their experiments and the findings reported from my laboratory on the basis of a β-glucuronidase response associated exclusively with processes of tissue growth. They have expended much effort employing *in vitro* studies in an attempt to prove that β-glucuronidase functions exclusively as an hydrolytic enzyme *in vivo*, and that there is no reason to consider that the enzyme has any direct connection with the metabolism or action of the estrogenic hormones.

It appears, at present, that the growth hypothesis of Kerr and Levvy, attractive as it may be, represents an oversimplification, at the very least, for the following reasons. We have been unable to confirm the allegedly high β-glucuronidase activities in the organs of new-born and infant mice, and we, along with investigators in other laboratories, have observed many instances where tissue growth was not accompanied by any alteration in β-glucuronidase activity of the tissue. We have also observed the reverse situation. Moreover, no convincing proof has been advanced by Kerr and Levvy which would indicate that β-glucuronidase does perform hydrolytically in tissue. In our own studies, we administered large amounts of β-glucuronidase intravenously in rabbits previously given two grams of menthol. There was no evidence of an appearance of free glucuronic acid in the urine. In a few instances, it appeared as if the animals had increased the output of conjugated menthol glucuronide.

How, then, may we visualize the synthesis of β-glucuronides, as this process may be catalyzed by β-glucuronidase? In my opinion, it is not obligatory to include considerations of high energy phosphate bonds and the complicated metabolite cycles, such as the tricarboxylic acid cycle, in the explanation of all biological syntheses, and it is my intention to postpone doing so for the β-glucuronide synthesis until all other possibilities have been excluded.

It is an elementary chemical principle that a reaction may be made to go to completion by the removal of one of the products of that reaction. We think usually of the formation of an insoluble compound or of a complex ion in this connection. Such model systems for the enzymatic synthesis of peptides were developed years ago by Bergmann and Fruton.

When the living cell is appraised as the site of enzyme action, it is apparent immediately that one is dealing with a multiphase system. The nucleus mitochondria and microsomes and the cell-membrane are in intimate contact with a colloidal solution containing proteins and many other constituents. The following are circumstances which would favor the synthesis of β-glucuronides by β-glucuronidase. One situation might be the formation of a poorly dissociated glucuronide-protein complex. Another would be the ability of the glucuronide to diffuse out of the cell into the extracellular fluid at a rate more rapid than that at which the reacting substances enter the cell. Still another might be the incorporation of the synthesized glucuronide into the relatively insoluble cell-membrane or the formed elements of the cytoplasm. It is possible, perhaps, that the physiologically important feature of β-glucuronidase is merely its high affinity for steroid glucuronides and that its role in direct synthesis is a minor one. It can be seen that the direct experimental proof of any one of these possibilities will be most difficult. It is to be hoped, however, that these views may provoke further investigation of this fundamental problem.

These considerations represent a modernization of the view we expressed in 1939 in that the synthesis of β-glucuronides in tissue is now regarded as taking place in a multicomponent system in the cell, with the enzyme β-glucuronidase representing an important constituent of this system.

One cannot exclude nor should one exclude the possibility that, under

some circumstances, β-glucuronidase may hydrolyze conjugates *in vivo*. Thus, the enzyme, which is found in saliva, gastric juice, tears, and vaginal fluid, existing as it does in a relatively homogeneous system and in solution, may very well hydrolyze β-glucuronides.

Finally, some questions should be raised. What is the nature and explanation of the increased tissue β-glucuronidase observed in uterus, liver, and kidney following administration of an appropriate steroid under the experimental conditions detailed previously? Is it due to an actual increment in the amount of enzyme protein, or is it the effect of an unknown enzyme activator? Experiments performed to answer these questions have not shown the presence of glucuronidase activating or inhibiting principles in the stimulated tissues.

What is the status of the "adaptation" hypothesis as a possible explanation of the steroid-induced enzyme increase in tissue? Recently, Buehler *et al.*[19, 20] reported that a strain of *Escherichia coli* was able to produce large amounts of β-glucuronidase when the organism was grown in a medium containing menthol-glucuronide. This may be taken as indicating that β-glucuronidase is capable, in the microorganism, at least, of adaptation to substrate. The level of β-glucuronidase in a given tissue and the relative ability of that tissue to respond enzymatically to steroid stimulation appears to be under the control of genes. These facts would seem to fit in with Spiegelman's concept[21] that "genes determine the potentiality for enzyme formation," which is a conclusion based on many complete studies of adaptive enzymes in microorganisms. In addition, there is the interesting behavior of desoxyribonucleic acid *in vitro* on purified β-glucuronidase, which may provide a clue concerning the mechanism of genic control. We do not know, of course, whether the possibly adaptive increase of enzyme is dependent entirely and directly on the administered steroid, or whether it results secondarily through effects of the steroid on the secretions of endocrine tissues, such as the pituitary or the adrenal.

In this paper, therefore, the enzyme, β-glucuronidase, is regarded as an enzyme catalyzing a primary reaction in the action of estrogen on the secondary sex tissues. The nature of this reaction is considered to be the catalysis in a multicomponent system of the synthesis of steroid glucuronides and probably also of non-steroid glucuronides involved in the manufacture of new protoplasm. The level of β-glucuronidase activity in the non-sex organs appears to be under genetic control, as is the relative ability of the liver to respond enzymatically to excess estrogen and of the kidney enzyme to be stimulated by excess androgen. At the present time, it is felt that the "growth" hypothesis of Kerr and Levvy represents an oversimplification. No evidence has been reported which would support their view that β-glucuronidase has an exclusively hydrolytic function *in vivo*. On the other hand, the view that β-glucuronidase is a component of a glucuronide conjugating system in tissue and is an enzyme which has the property of "adaptation," first formulated in this laboratory, does explain many of the observations reported, although not all of them. Direct evidence in support of this concept is lacking and will be difficult to collect. For the

benefit of investigation aimed at elucidating the exact *in vivo* function of β-glucuronidase, it is my opinion that both of these hypotheses should serve the function of suggesting new experiments, and they should not be elevated to the status of "theories." Clearly, the need here is for more clean-cut experimental facts and not for the elaboration of additional hypotheses.

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THE STEROIDAL HORMONES AND TISSUE β-GLUCURONIDASE AND ESTERASE*

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The data reviewed in this paper are organized to demonstrate: first, a possible genetic influence on tissue levels of glucuronidase; second, the inverse relationship between changes in glucuronidase and esterase in tissues and blood; third, the effects of a number of steroidal hormones on the activity levels of these enzymes in tissues and blood; and fourth, an apparent relationship between the serum level of β-glucuronidase and the proportion of urinary steroids conjugated as glucuronides.

A. *The Genetic Influence on the Relative Glucuronidase Levels of Tissues.* Morrow, Greenspan, and Carrol suggested in 1949[16] that the relatively low liver glucuronidase of the C_3H strain of mice might be genetically controlled. The experiments which Dr. Bittner and the author[4] have carried out on the relative glucuronidase and esterase levels in the mammary tissue of a number of stocks of mice lend support to this thesis.

(1) A marked difference in the glucuronidase activity of the mammary tissue in different mouse strains was found (FIGURE 1). Thus, there were four units of enzyme activity in the non-tumorous tissue of the Andervont C_3H strain of mice, while corresponding tissue in the C stock showed 120 units of activity per mg. tissue N.

(2) Corresponding relative glucuronidase levels are to be observed for a number of tissues in the different mouse strains. Thus, enzyme studies on blood serum (see TABLE 1) showed that the serum glucuronidase of the Z mice is only about 20 per cent as high as that of the A strain, while the glucuronidase activity of the non-tumorous mammary tissue for the Z strain was about 28 per cent of that of the A strain (FIGURE 1). In this connection, it might also be pointed out that Morrow *et al.*[16] reported the liver glucuronidase activity of C_3H mice to be only about 10 per cent of that of the A mice, while we observed a correspondingly low ratio of about five per cent for non-tumorous mammary tissue.

(3) Particular support for the theory that the glucuronidase activity levels of mice are genetically determined is lent by our observations on the ZD_8F_1 mice. These mice are hybrids obtained by crossing Z female mice with D male mice. They showed glucuronidase levels about the same as those of D mice but about 3–4 times as much as those of the Z mice.

While the final proof of a possible genetic determination of the glucuronidase activity in mice must await breeding experiments, the possibility of such an influence emphasizes the danger of drawing general conclusions from experiments carried out on one stock of mice. This danger was particularly emphasized in the experiments just reported by Dr. Fishman on the effects of estrogen on the liver glucuronidase of mice.

* These studies were aided by grants from the National Cancer Institute of the National Institutes of Health, Public Health Service, from the American Cancer Society, and from the University of Minnesota Graduate School.

B. *The Relationship between Glucuronidase and Esterase Changes in Tissues.* It has further been found that for all strains there is a considerable

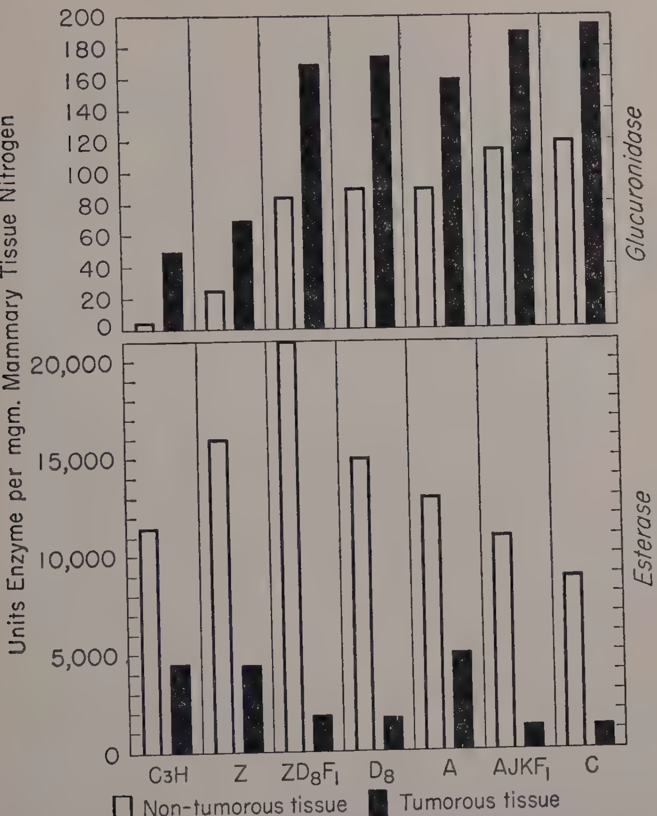

FIGURE 1. The glucuronidase and esterase activities of mammary tissues in a number of mouse strains (from the data of Cohen and Bittner[4]). The strains and F_1 generations shown all had mammary tumors and are in order: C_3H (Andervont), Z(Bittner C_3H), ZD_8F_1 hybrids (Z females x D_8 male), D_8 (dilute brown stock, Bittner subline D_8), A, $AJKF_1$ (A females x JK male), and C.

TABLE 1

SERUM GLUCURONIDASE (G) AND ESTERASE (E) IN MICE

Strain	*No. of assays*	*Units G/100 cc. serum (average values)*	*Units E/cc. serum (average values)*
Z	4	360	27,000
C57	6	1,150	26,000
A	8	2,000	38,000

elevation in the glucuronidase content of tumorous mammary tissue as compared to that of the non-tumorous tissue in the same mouse. While relationships between tumor developments and the steroid hormones have been suggested, no conclusive proof of such relationships is as yet available. Postulations of the possible role of the steroidal hormones in the enzyme

changes observed in these mouse mammary tumors will, therefore, not be discussed at this time. The data of FIGURE 1 do, however, emphasize that the increased glucuronidase in the tumors is associated with a marked re-

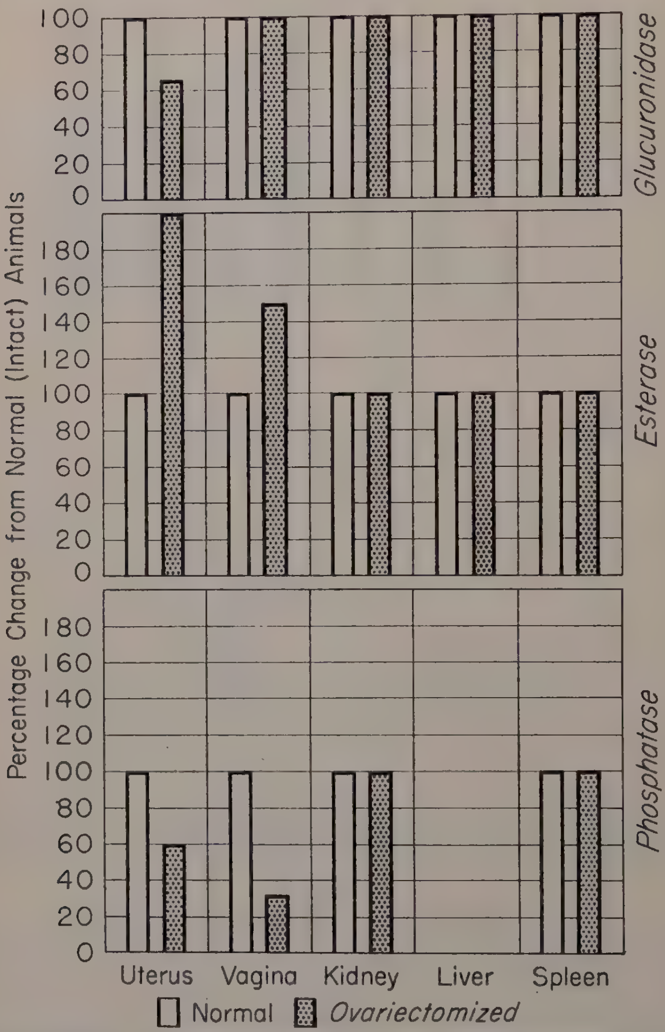

FIGURE 2. The effect of ovariectomy on the enzyme activities of tissues in mice (from the data of Harris and Cohen[12]).

duction in the esterase levels of the tissue. In practically all of our studies on the effects of steroidal hormones on blood and tissue glucuronidase in the human, as well as in mice, a similar inverse relationship between the glucuronidase and esterase responses was observed.

Thus, factors responsible for an elevated glucuronidase also effect a reduced esterase, while a decrease in the glucuronidase activity of a tissue

has been found to be associated with an increase in the esterase activity of that tissue. Our studies on the time relationships of these changes are not yet complete, so it is as yet impossible to say whether a change in the concentration of one enzyme precedes a change in the concentration of the other, or whether both are changed simultaneously. It is hoped that such information might be useful in the formulation of a hypothesis regarding this inverse relationship between changing glucuronidase and esterase levels.

C. *The Effect of Steroidal Hormones on the Activity Levels of Glucuronidase and Esterase in Tissues and Blood.* (1) *Estrogen.* In the preceding paper, Dr. Fishman has summarized the data relating glucuronidase activity and estrogens. Some of the confirmatory results obtained in our laboratory will be briefly repeated in order to emphasize additional points.

(a) The effects of ovariectomy on the enzymic activity of mouse tissues are reviewed in FIGURE 2. It is readily seen that, as previously pointed out, ovariectomy causes a reduction in the glucuronidase level in the uterus.

TABLE 2
ENZYME ACTIVITIES OF MOUSE VAGINAL SMEARS

Smears from:	*Units G/mg. N*	*Units E/mg. N*	*No. leukocytes/mg. N*
Intact animals	17	460	2.5×10^{-6}
Ovariectomized	21	830	6.7×10^{-6}
Ovariectomized + estrone	6	500	0.33×10^{-6}

The other sexual tissue examined, the vagina, failed to show a reduction in β-glucuronidase. This observation is difficult to reconcile with either the "metabolic conjugation" theory of Fishman[8] or with the "growth" theory of Kerr, Levvy, and Campbell[13] for glucuronidase in tissues. We have recently obtained evidence, however, to indicate that the tissue cells proper of the vagina probably undergo some reduction in β-glucuronidase activity on castration. This evidence is based upon assays carried out on the vaginal smears (obtained by means of the lavage technique) of these mice (TABLE 2). The marked reduction in the leukocyte count and in the glucuronidase activity of the smear following estrone treatment of ovariectomized mice suggests that a low vaginal glucuronidase may be masked by a relatively high glucuronidase activity of the polymorphonuclear leukocytes which abound in the vaginal tissue of the castrated mouse.

Other observations which may be made from the data summarized in FIGURE 2 are (1) the failure of the non-sexual tissues examined (kidney, liver, and spleen) to show any enzyme changes resulting from ovariectomy, (2) the increased esterase values in the uterus and vagina following castration, and (3) a reduction in the alkaline phosphatase of both the uterus and vagina in the ovariectomized mice. On the basis of these and of other experiments to be discussed, it would seem that the alkaline phosphatase activity of a tissue more closely parallels growth than does the glucuronidase

content as suggested by Kerr, *et al.*[13] The restorative effect of suitable doses of estrone on the enzyme changes effected by ovariectomy is shown in FIGURE 3. It is also to be noted that the administration of estrone effected a considerable response in the relatively immature mammary tis-

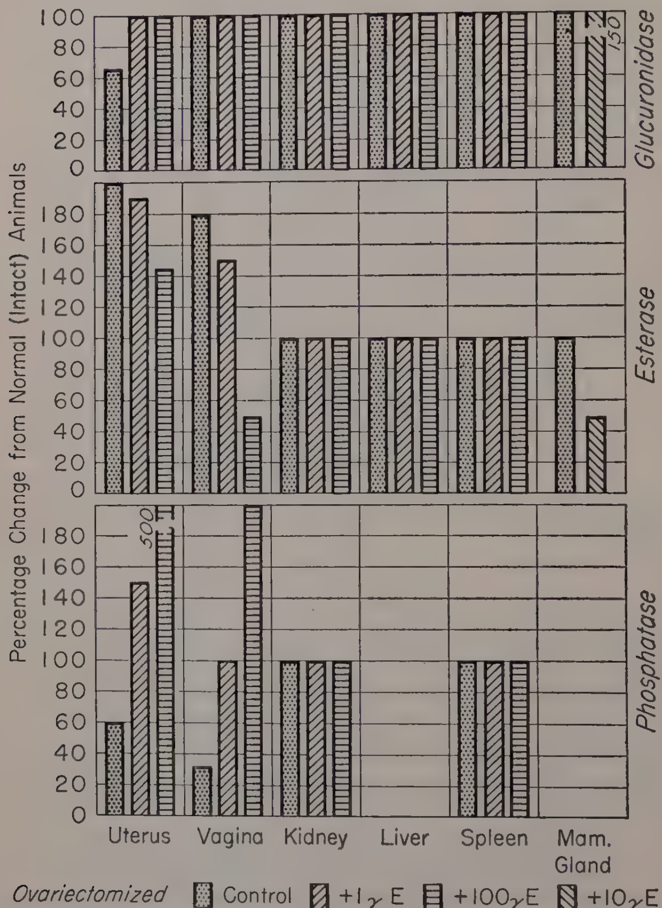

FIGURE 3. The effect of estrogen on the enzyme activities of tissues of ovariectomized mice (taken in large part from the data of Harris and Cohen[18]).

sue. Here, too, changes in the phosphatase level of a tissue seem to parallel its growth response.

The administration of estrogens also causes an increase in the β-glucuronidase of the blood serum of humans. Such an increase was first observed for post-menopausal patients with advanced breast cancer and on estrogen therapy (FIGURE 4). The patients treated with either diethylstilbestrol or with ethinyl estradiol showed maximum responses of almost 100 per cent of the pre-therapy serum glucuronidase levels. The stilbestrol treated patients, however, showed a more slowly elicited response than did the estradiol treated patients. The data shown in FIGURE 5 illustrate the

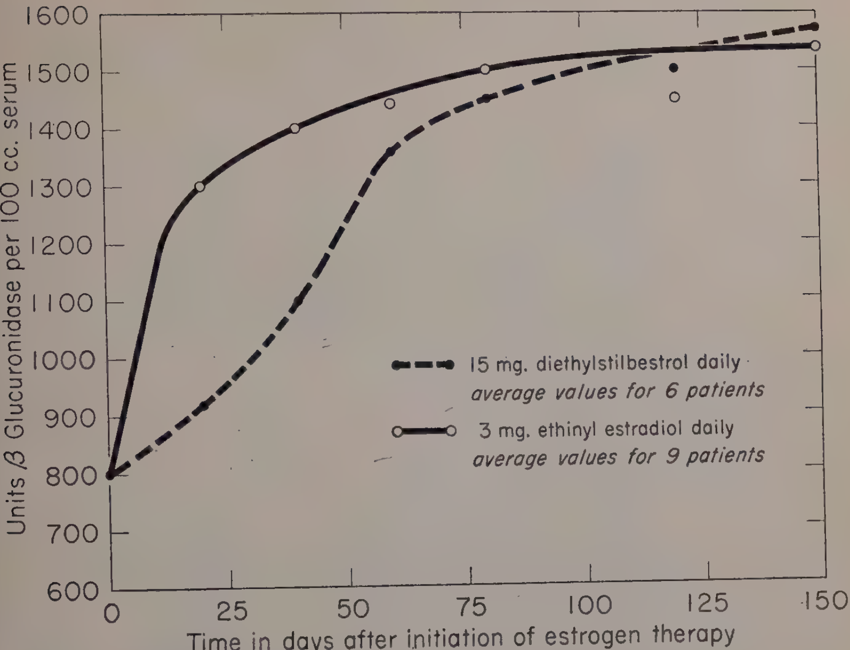

FIGURE 4. The effect of estrogen therapy on serum glucuronidase of patients with breast cancer (from data of Cohen and Huseby[5]).

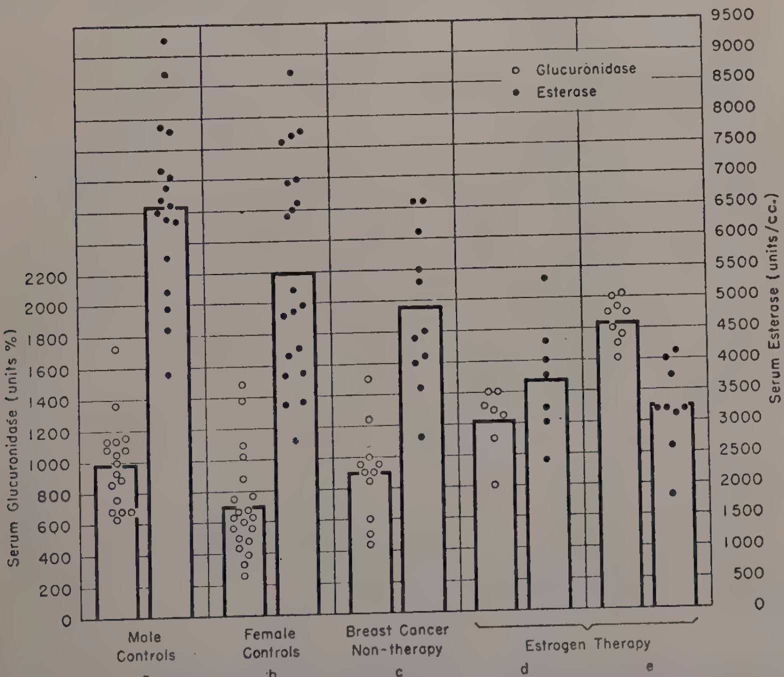

FIGURE 5. The effect of estrogen therapy of patients with breast cancer on serum glucuronidase and esterase (reprinted from COHEN, S. L. & R. A. HUSEBY. 1951. Proc. Soc. Exp. Biol. & Med. 76: 304).

decreasing serum esterase which accompanies the rising glucuronidase in these patients. Fishman *et al.* have recently reported a similar increased serum β-glucuronidase in normal post-menopausal women receiving stilbestrol (5 mg./day).[10]

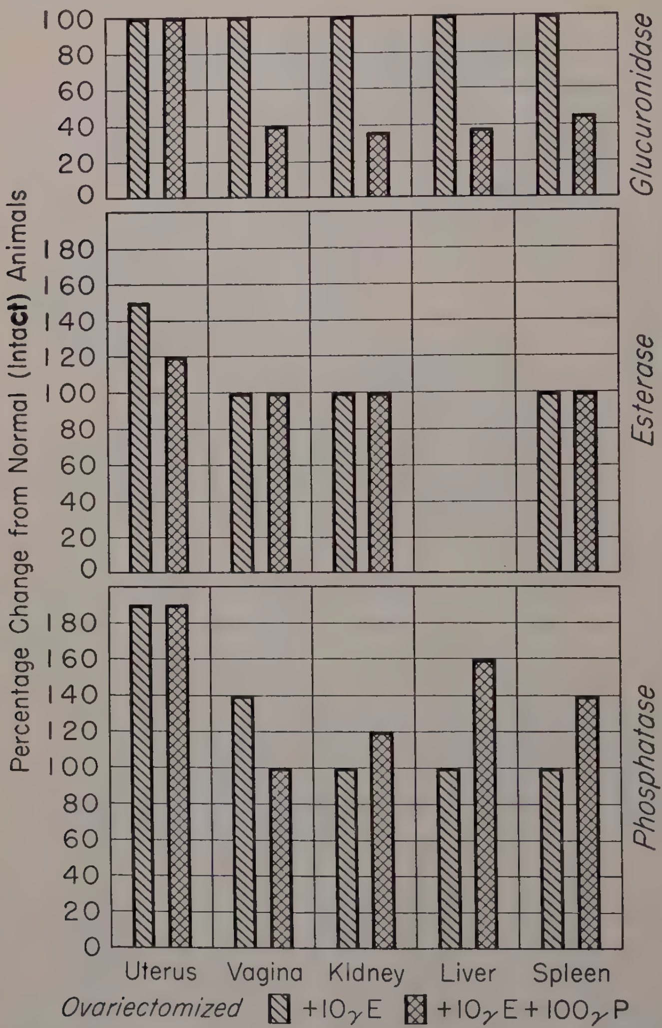

FIGURE 6. The effect of progesterone on enzyme activities of tissues in mice (from the data of Harris and Cohen[12]).

(2) *Progesterone.* The effects of progesterone on the enzyme activities of estrone-sensitized overiectomized mice are shown in FIGURE 6. A marked reduction was observed in the glucuronidase activity of all the tissues examined except the uterus. Some preliminary experiments indicate that mammary tissue also shows a reduced glucuronidase and elevated esterase when subjected to the action of progesterone. These changes in β-glucu-

ronidase levels are impossible to reconcile with the theory of Kerr *et al.*[13] directly relating the glucuronidase activity of a tissue with its mitotic activity. This is particularly so if the alkaline phosphatase level of a tissue is related to its degree of growth, since progesterone had no corresponding reducing effect on the phosphatase activity of any of the tissues studied. The scarcity of mitotic figures in the kidney also strongly contraindicates a relationship between the glucuronidase and mitotic activities of a tissue. There was a tendency for an increasing esterase in the responding tissues. The relatively small esterase changes in some cases might be due to a time factor in which the esterase response requires a longer period of time to manifest itself than does the reduced β-glucuronidase response.

(3) *Testosterone.* In five of six patients with advanced breast cancer, testosterone effected no significant change in the level of serum glucuronidase. A definite increase in the serum glucuronidase occurred in the sixth patient studied. This patient, however, died several months after the initiation of therapy, and it is therefore possible that the rising glucuronidase may actually have been associated with an increasing condition of stress (see below) rather than with the testosterone therapy.

(4) *Adrenal Steroids.* We have recently obtained evidence that steroidal hormones of the adrenal cortex may cause a rise in the serum β-glucuronidase. Studies on the effects of the administration of cortisone to two female patients with pseudohermaphroditism showed significant serum enzyme changes in both (TABLE 3). It should be emphasized that in these two patients at least 1–2 weeks of continuous cortisone therapy were required before these serum enzyme changes were manifested. It will be recalled that estrogen therapy also has a relatively long latent period before eliciting significant changes in the serum glucuronidase levels (see FIGURE 4). Additional studies on the enzymic effects of cortisone and related steroids, particularly in patients being treated for other conditions, are at present being undertaken. Indirect evidence of alterations effected in Cushing's disease and as a result of stress lend support to the suggestion of an adrenal cortical influence on the serum glucuronidase level. Thus, in three of four patients with Cushing's disease and in three patients subjected to surgery, there were marked elevations of the serum glucuronidase levels. Two patients with breast cancer and in considerable stress also showed an elevated serum glucuronidase. All of the four cases of pregnancy thus far examined showed a marked elevation in the activity of serum glucuronidase in association with pregnancy. (The effect of pregnancy on serum glucuronidase was first reported by Fishman[9] and by McDonald and Odell[15] in 1947.) The high estrogen formation during pregnancy,[7] however, might well be a more important factor in raising the blood glucuronidase than the increased corticoid production which occurs in this condition.[17]

It is also to be seen from the data shown in TABLE 3 that, in all cases where an increased serum glucuronidase in a patient was effected, an associated reduction in the serum esterase level also occurred.

D. *The Relationship between Serum β-Glucuronidase and the Proportion of Steroids Excreted as Glucuronides.* The relative amounts of corticoids

(formaldehydogenic) and ketosteroids released by HCl[1, 3] and by a concentrate of calf spleen β-glucuronidase[3] from a number of urine specimens have been determined. The relatively mild acid conditions which must be

TABLE 3

EFFECT OF ADRENAL CORTEX AND STRESS CONDITIONS ON SERUM GLUCURONIDASE (G) AND ESTERASE (E)

Condition	*Patient*	*Sex*		*G(units %)*	*E(units/cc)*		*G(units %)*	*E(units/cc)*
Cortisone therapy	C. D.*	F	Pre-therapy	550	5800	100 mg. cort./day	1500	4200
Cortisone therapy	E. H.*	F	Pre-therapy	570	4900	50 mg. cort./day	800	3600
Cushing's disease	C. E.	F		2000	5700			
	S. R.	F		1800	3800			
	L. F.	F		2200	3700			
	R. L.	M		340	4200			
			Average	1600	4400			
Addison's disease	H.	F		520	3900			
	D.	M		660	3700			
	J. N.	M		600	5600			
	O. T.	M		360	4600			
	C. K.	F		270	5400			
	C. P.	F		440	5300			
			Average	470	4700			
Surgical patients	N. D.	F	Pre-surg.	1100	3000	Post-surg.	1800	1700
	H. L.	M	Pre-surg.	780	5900	Post-surg.	1400	4400
	W. A.	M	Pre-surg.	960	6150	Post-surg.	1500	4600
			Average	950	5000		1600	3600
Breast cancer patients in considerable stress	M. K.	F		1900	2700			
	M. H.	F		1300	4700	1 month later	1800	3100
Pregnant patients	R. H.		Late preg.	1200	4600	Pre-preg.	330	9000
	A. C.		Pre-part.	1800	3500	Post-part. (8–10 d.)	1000	4200
	E. F.		Pre-part.	1700	4400	Post-part.	500	5200
	M. B.		Pre-part.	1800	3500	Post-part.	1200	4800
			Average	1600	4000		750	5800

* Pseudohermaphrodite.

used in order to limit corticoid destruction hydrolyzes only a small proportion of steroid conjugates. Thus, no method is yet available for the complete hydrolysis of all of the corticoid conjugates in urines. On the other hand, the "total" ketosteroid content of urines may be approximately determined,[2] and it is thus possible to determine the precentage of this "total" ketosteroid content of urine that is released by glucuronidase preparations. A comparison of the values thus obtained with the levels of glucuronidase in serum obtained during the urine collection period is shown in TABLE 4.

While the number of cases studied in each group is not large, it is readily seen that in all groups an increased serum glucuronidase is associated with an increased proportion of glucuronide conjugated ketosteroids excreted into the urine. Of the 34 patients studied, only three exceptions to this general observation were found (one case in each of the breast cancer, Cushing's, and Addisonian categories). It should be pointed out that patients exposed to the stress of surgery and assayed two days after the surgery showed only a relatively small increase in serum glucuronidase and no increase in the proportion of ketosteroids conjugated as glucuronides. The peak serum enzyme response was not shown until about the 5th post-surgical day, and, in both cases studied so far at this post-surgery interval,

TABLE 4
THE RELATIONSHIP BETWEEN SERUM GLUCURONIDASE AND URINARY KETOSTEROID GLUCURONIDE LEVELS

Condition	*No. of patients*	*Units % serum glucuronidase (G) (average values)*	*Per cent "total" Ks. conjugated as glucuronides (average values)*
Pregnancy			
Pre-partum (2–6 weeks)	6	1800	64
Post-partum (8–10 days)	3	900	40
Adrenal Dysfunction			
Addison's disease	3	460	39
Hypopituitary	2	680	34
Cushing's disease	4	1600	62
Cortisone treated	2		
without therapy		560	49
with therapy		1200	62
Breast Cancer Patients			
with serum G below 1000 units %	10	820	39
with serum G above 1000 units %	2	1800	66
Surgery Patients	2		
Pre-surgery		860	31
Post-surgery (5 days)		1500	50

an increased proportion of glucuronide conjugated ketosteroids was demonstrable.

These data tend to indicate a relationship between the serum concentration of β-glucuronidase as measured by a hydrolytic procedure (the substrate employed in our studies was phenolphthalein glucuronide[11]) and steroid glucuronide synthesis within the organism. The suggestion of Levvy[14] of the existence of a specific synthetic glucuronide enzyme system would thus be obviated. Our experiments, however, do not rule out a possible parallel increase in the concentration in the body of such a specific glucuronide synthesizing system with the hydrolytic glucuronidase. Such a postulation, however, seems at present to the author unnecessarily complicated.

Summary

The possible influence of genetic factors in determining the relative β-glucuronidase levels of mice suggests the use of caution in making general

conclusions regarding enzyme responses observed in only one strain of animal. Data indicating the effects of various steroidal hormones on glucuronidase and esterase levels in the organism are presented. Estrogens in mice (Wistar strain) have been observed to cause an increased glucuronidase of the secondary sexual tissues examined (uterus, mammary gland, and probably also vagina), but no effect was observed in the non-sexual tissues (kidney, liver, and spleen). Progesterone causes a reduction in the glucuronidase levels of both sexual and non-sexual tissues in these mice. In humans, an elevation of the serum β-glucuronidase is associated with estrogen administration (at least for post-menopausal women) and with increased amounts of circulating glycogenic adrenal cortical steroids. In all observations on both mice and men, an inverse change in the esterase was associated with glucuronidase variations. A relationship between the level of serum glucuronidase in humans and the proportion of urinary ketosteroids excreted as glucuronide conjugates is indicated.

Acknowledgments. The experiments reported in this paper were made possible by the cooperation of Drs. R. Huseby, E. Flink, B. Zimmerman, and I. McQuarrie of the University of Minnesota Hospitals in the procurement of blood and urine specimens and by the patience and skill of Florence I. Bowers, Karol Friedman, and Barbara Magnus in the carrying out of the many assays involved.

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THE INFLUENCE OF ADRENALECTOMY AND CORTISONE TREATMENT ON ENZYMATIC REACTIONS IN RAT TISSUES

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It is painfully evident that, at our present state of knowledge, the "mode of action" of hormonal agents, such as cortisone, is almost completely obscure. In such a situation, it is desirable not only to follow those specific clues which may be happened upon, but also to undertake systematic studies of those portions of metabolism accessible to such investigations in an effort to uncover actions of hormones which are not indicated by superficial observation.

In the case of the hormones of the adrenal, there is an additional factor which requires consideration. Upon total adrenalectomy, in the rat, provided no accessory adrenal tissue exists, the animal is totally deprived of certain products of the adrenal. Yet, if provided with an adequate salt balance, such an animal will not only survive, but grow to maturity and exhibit all of the functions associated with the life of a rat, save only that, to a certain extent, susceptibility to some forms of stress will be increased. Such a rat can make liver and muscle glycogen; it can do work; it can live, and grow, and make a variety of adjustments to its environment. Certainly, with adequate salt intake, whatever is missing is not essential to life. From what deficiency does such an animal then suffer? Why is it unable to respond to stress in an adequate manner? What do the hormones do to permit it to respond in this way?

Phrased in this manner, the enzymatic approach to the problem of cortisone action becomes one of probing into a complex problem with what are very probably quite inadequate tools for the magnitude of the subject. In fact, we do not at this stage know whether the basis of the action of cortisone lies in the failure, either quantitative or qualitative, of a particular enzyme system, or of a group of enzyme systems or, in fact, whether its basis is enzymatic at all. So our object, at this time, is to determine whether or not adrenalectomy or cortisone treatment will alter tissue enzymes. When and if a pattern of alteration is established, it may be possible to discern its significance. At any rate, it should contribute to the problem of whether the basis for the action of cortisone is indeed enzymatic.

The ability to withstand, or to adjust rapidly to, conditions of stress may have, as its enzymatic basis, the ability to mobilize energy stores rapidly, *i.e.*, to draw upon reserves of energy and matter not normally an important part of the metabolic machinery. The relations of cortisone to fat, protein, and carbohydrate metabolism are amply documented in the physiological literature. All of these materials have as their terminal point of metabolism some aspect of the citric acid cycle. Since they are all influenced by cortisone, it seemed reasonable to start at this common meeting ground of metabolism and to note what effects, if any, cortisone exerted on this system. From this point, one could branch out into the various pathways leading

into this system from fat, protein, and carbohydrate and eventually come upon those in which the hormone is fundamentally concerned, if, indeed, its action is basically upon tissue enzymes.

This is not, of course, the only approach available. The systematic study of the converging points of metabolism, however, might uncover effects and mechanisms which might otherwise remain obscure. Indeed, such effects were found. I do not suppose that anyone was more suprised than we to find effects of adrenalectomy and cortisone treatment on the oxidation of citric acid cycle intermediates. Typical data for a few substrates obtained with kidney homogenates are given in TABLE 1. The effects were not large in most cases. In fact, for succinate, or for pyruvate plus fumarate, they were not significant. For α-ketoglutarate, however, they were quite marked. Glutamate and proline are included as examples of amino acids one and two steps, respectively, removed from the cycle, and it is of interest to note that, in this case, apparently, the farther one went away from the cycle, the greater was the effect of adrenalectomy. Since all of these effects could be restored by treatment of the adrenalectomized animal with cortisone (all adrenalectomized animals received saline, whether treated with cortisone or not), it appeared that the significant adrenal factor lacking was cortisone.

TABLE 1

OXIDATION OF VARIOUS SUBSTRATES BY ISOTONIC KIDNEY HOMOGENATES

*Substrate**	*Number of animals*	*Treatment of animal*		
		Normal	*Adrex.*	*Adrex. & cortisone*†
N		Q_{O_2}	Q_{O_2}	Q_{O_2}
None	6	5 ± 1	3 ± 1	3 ± 1
Pyruvate & fumarate	9	44 ± 6	36 ± 5	38 ± 3
α-ketoglutarate	12	44 ± 3	33 ± 2	41 ± 2
Glutamate	9	35 ± 3	25 ± 1	38 ± 2
Proline	15	25 ± 4	10 ± 2	26 ± 4

* All substrates at 0.016 M.; adenylic supplement at 0.001 M.
† Cortisone treatment: 1 mg. Cortone intraperitoneally per day.

Upon further study, it was demonstrated that effects of adrenalectomy and cortisone treatment apparent in this table were fundamentally due to two alterations. One concerned the proline system. The other concerned the adenylic acid-ATP system. We shall consider these effects separately.

At this stage, it was perhaps sensible to study proline, since there seemed to exist some vague relationship between collagen and the action of cortisone and since, of all of the proteins of the body, collagen is characterized by its high proline:hydroxyproline content. Even now, after all the time that has gone into the study of this phenomenon, I am not so sure that there may not be more significance to this effect on proline than we can now establish. Hydroxyproline is oxidized by essentially the same system, and the phenomena which I shall attribute to proline hold for hydroxyproline as well.

There is no need, at this time, to go into the details of the proline study, since they have recently been published.[1] A brief summary will suffice here. The time after adrenalectomy required for the loss of proline oxidizing ability of the kidney is three days. If cortisone is administered over this interval, 0.5 mg. per rat per day is adequate to maintain this function. This is roughly 2–3 mg. per kilogram. If the proline oxidation is allowed to decrease, and thereafter cortisone treatment is instituted, about a week of such treatment is required before the activity is completely restored. The mechanism of proline oxidation in kidney homogenates from both the adrenalectomized and cortisone-treated rats is that described by Taggart and Krakaur[2] and illustrated in FIGURE 1. In no case was *in vitro* acti-

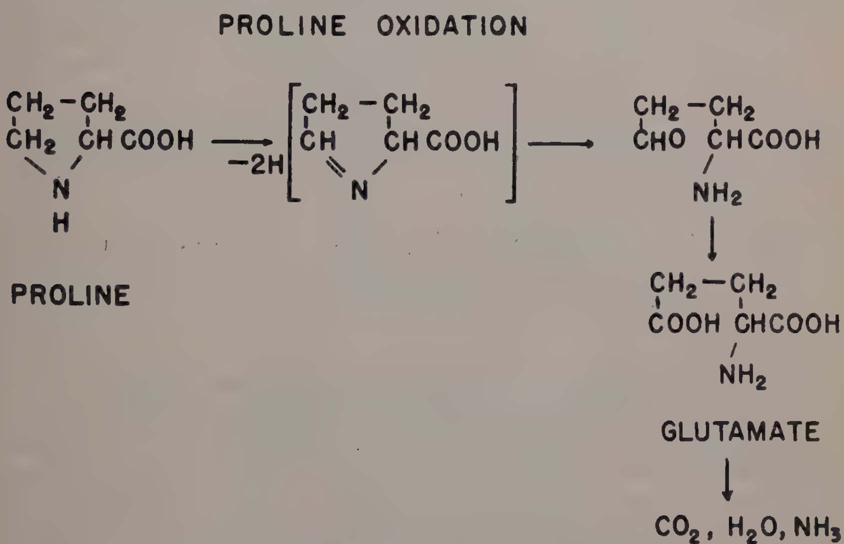

FIGURE 1. Mechanism of proline oxidation.

vation of this system observed by the addition of cortisone in various forms or by the addition of heat or cold inactivated preparations from cortisone-treated animals. On the basis of this type of information, one must necessarily conclude that the role of cortisone is in the formation rather than in the activation of the enzyme. This conclusion must stand until such time as data supporting any other alternative may be found. The only condition under which cortisone was effective in maintaining or restoring this system was when it was supplied to the whole animal. Desoxycorticosterone was not effective. The proline oxidation system could be used for the detection and assay of cortisone, but, since other methods are available, it has not been used for this purpose.

With the exception of the adenylic effect to be described in a moment, no other change in enzymatic systems was detected in kidney homogenates. Included in these studies were the first steps in the entrance of fat, protein, and carbohydrate metabolic products. There was no change, for example,

in transaminase, in octanoic or acetoacetic oxidase, or in the oxidation of amino acids other than proline or hydroxyproline. In fact, the effect on proline appeared to be quite specific and would suggest that the relationship of cortisone to protein, fat, and carbohydrate metabolism is to be sought at stages somewhat farther removed from the terminal point represented by the citric acid cycle. There remains, however, the effect on proline, which requires some further comment.

Whenever one is confronted with the situation that the apparent amount of an enzyme in a given tissue is influenced by an agent external to the tissue, there is always the possibility that the effects observed are indirect. They may, for example, be dependent upon the amount of available protein in a tissue, or they may be a reflection of some more deep-seated change. In the case of proline oxidation by the kidney, however, this is apparently not the case. The rate at which a kidney homogenate from a normal animal will oxidize proline *in vitro* is not dependent upon its nutritive state, upon its age or weight (within the rather broad limits measured), or upon any factor that we have yet come upon, save only the presence of cortisone. Increasing the dosage of cortisone tenfold beyond that required for maintaining the system does not increase the proline oxidase. The system is perfectly normal in rats deficient in pyridoxine, vitamin B_{12}, thiamin, pantothenic acid, or riboflavin. The last two are of particular interest, since there is evidence of some sort of adrenal insufficiency when these vitamins are lacking. Apparently, however, this deficiency is not due to the lack of cortisone.

Upon adrenalectomy, the kidney Q_{O_2} on proline drops from 25 to 10. In the adrenalectomized rat maintained for three months on saline, the Q_{O_2} is still 10. Various methods have failed to reduce the proline oxidation rate below this point. There thus appears to be a certain minimum below which the rate of proline oxidation will not drop and a certain maximum beyond which, even with excess of cortisone, it will not rise. Furthermore, the action of cortisone is exerted only between these limits. I have the feeling that this aspect of the action of cortisone has some fundamental significance and may represent a type of action common to a variety of hormones when studied at the enzymatic level. The causes of this phenomenon, however, are not clear to me.

Since I have raised the question of significance, it is well to point out one other matter with respect to proline. It is possible, but, to my mind, not very probable, that the derangement in the metabolism of but one amino acid could exert such a profound change in the overall metabolism of the body as to cause a variety of symptoms to appear. Curiously, however, while the oxidation of proline is so closely under the control of cortisone in the kidney, it is entirely independent of this hormone in the liver. As far as we have been able to determine, the mechanism of proline oxidation in the liver and the kidney are the same; presumably, the enzymes are also the same. We should point out that the proline oxidase of either tissue has thus far resisted isolation in anything more than preparations containing the citric acid cycle complex, so that subtle differences between the enzyme from the two organs may be obscured. These facts, however, make

any generalized interpretation of the significance of proline oxidase of the kidney to the mode of action of cortisone somewhat tenuous.

The other alteration in the kidney enzymes so far observed is an increased requirement for adenylic acid in order to maintain the maximum rate of oxidation. This effect does not appear as rapidly as the lowered ability to oxidize proline, usually requiring a week before it is fully evident. The details of this reaction are complex and require a much more detailed exposition than seems warranted here. Thus, since the details have recently been published,[3] we shall only say that this increased requirement for adenylate is the cause of the decreased oxidation in homogenates from

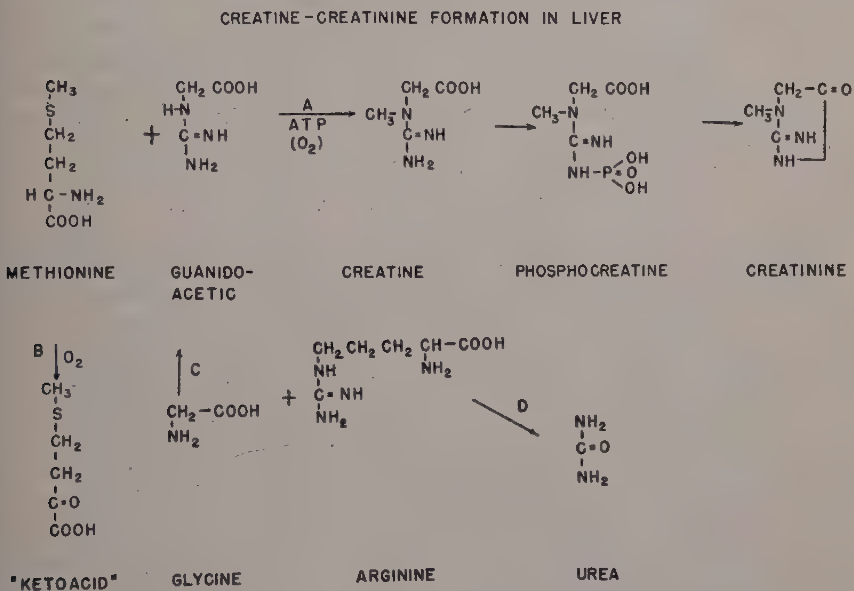

FIGURE 2. Reaction leading to creatine formation in liver.

adrenalectomized animals of substances such as α-ketoglutarate, as previously shown.

So far, we have discussed in detail the effect of cortisone upon the proline system in the kidney, pointing out that the same enzyme apparently was not under the control of cortisone in the liver. Because of the glycogenic effect of cortisone, it must be acting in the liver. Indeed, we have been able to show that another enzyme, D-amino-acid oxidase, is under the control of cortisone in the liver, but not in the kidney. We were led to this enzyme in a rather curious way, which will bear a brief discussion.

As mentioned, it was originally felt that the ability to adjust to stress might reside in the ability to draw upon reserves of energy and matter not normally a quantitatively important part of the metabolic pathways. Having examined the transformations of matter, at least in the terminal respiration systems, we simultaneously examined the energy relationships. We found[4] no effect of adrenalectomy or cortisone treatment on the phosphocreatine of muscle, kidney, or liver, upon the ATP, or upon the ability

of homogenates to generate energy-rich phosphate. In the course of this study, however, we had occasion to examine the system in the liver forming creatine from methionine and guanidoacetate, and here we obtained an apparent effect of adrenalectomy and cortisone treatment. Some of the reactions involved are given in FIGURE 2. The system forming guanidoacetate from arginine and glycine is included, since adrenalectomy has long been associated with a decreased arginine metabolism. Upon further study, however, it was found[5] that the creatine which we thought we were measuring was in reality the keto-analogue of methionine; that it was formed only from D-methionine; and that, in fact, the enzyme under the control of cortisone in the liver was D-amino acid oxidase.

Essentially the same picture holds for this liver enzyme as cited for the proline oxidase of kidney, with one general exception. Whereas proline oxidation was not influenced by alterations other than cortisone, the D-amino-acid oxidase of liver is subject to dietary alterations, to fasting, *etc.* The quantity present in liver may be a reflection of available protein in this organ, or in the availability of materials from which the enzyme can be constructed. If so, then cortisone controls the availability of these materials. If this were the case, however, one would expect two other corollary matters to occur. First, one would presume that there would be increased availability throughout the body, and that therefore the kidney would have more of such building materials and that it too would form more D-amino acid oxidase. This does not happen. The D-amino acid oxidase of the kidney is uninfluenced by adrenalectomy or cortisone treatment in the same animal in which the liver enzyme drops on adrenalectomy and is restored to normal by cortisone treatment. Second, if cortisone is concerned solely with the availability of materials in the liver, one would suppose that other enzymes would also alter in the liver—succinoxidase, for example, whose content can be altered by dietary changes. Yet, this does not happen. Of the enzymes studied, only D-amino acid oxidase is controlled by cortisone.

This leads us into what is indeed a most curious situation. The oxidation of proline and hydroxyproline, and little else, is under the control of cortisone in the kidney, but not in the liver. The oxidation of D-amino acids is under the control of cortisone in the liver but not in the kidney. In both cases, the cortisone acts only between certain limits, and in both cases the effect of cortisone is apparently upon the amount of enzyme present rather than upon its activation. The differential effect observed from tissue to tissue and the restriction of cortisone activity to certain limits of enzyme content would appear to be important factors in the action of cortisone on tissue enzymes.

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THE INFLUENCE OF STEROIDS ON CEREBRAL METABOLISM

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Selye's[1] observation, in 1941, that certain steroids produce anesthesia in experimental animals has stimulated interest in the effects of hormones upon cerebral metabolism. Michaelis and Quastel[2] suggested that the action of anesthetics is due to interference with the oxidation of carbohydrate substrates. Their data indicate a possible site of this inhibition at some level between flavoproteins and cytochrome oxidase. In their review in 1944, Jensen and Tenenbaum[3] were forced to conclude that reports of steroidal relationships to specific enzyme systems *in vitro* were lacking. McShan and Meyer, among others, have, to a large degree, filled this gap in the years which have intervened.[4-6]

I. EFFECT OF STEROIDS ON THE RESPIRATION OF RAT BRAIN IN VITRO

A. Comparative Inhibitory Effect of Stilbestrol and Various Steroids. These observations provided the basis for investigation of the effects of diethylstilbestrol and several steroids on the respiration of cellular homogenates of rat brain.[7] The influence of these compounds upon the oxidation of glucose, succinate, and pyruvate was studied by the direct method of Warburg. The effects on the oxygen consumption of cellular homogenates using a glucose substrate are shown in figure 1. With the exception of diethylstilbestrol, the steroids inhibit respiration in the presence of glucose in parallel to their anesthetic potency as determined by Selye.[8] Cholesterol, which is devoid of hormonal action *in vivo*, had little effect. In common with other anesthetics, the steroids had little effect upon the oxidation of succinate, diethylstilbestrol again excepted (table 1).

Desoxycorticosterone was the most potent steroid of the group tested. These results have been confirmed by Hayano, Schiller, and Dorfman[9] and are in accord with the report of Tipton[10] that whole adrenal cortical extract and crystalline corticosterone depressed the oxygen consumption of rat brain tissue. The work of Hayano, Schiller, and Dorfman[9] has shown that the oxidative activity affected by the steroid resides, for the most part, in the centrifugate.

B. Site of Inhibitory Action. Studies have been undertaken to determine a site of hormonal action on the main line of biological oxidation which could result in an inhibition of oxygen consumption.[7] Methylene blue (6×10^{-5} molar) reversed stilbestrol-induced inhibition of the succinoxidase system of rat brain. This observation supports the work of McShan and Meyer,[4] who found that the inhibition of rat liver and pituitary succinoxidase systems produced by estrogenic stilbenes was correlated with

* With the cooperation of the Division of Neurological Surgery of the University of California School of Medicine, and the Langley Porter Clinic of the State of California Department of Mental Hygiene, San Francisco, California.

the number of terminal phenolic groups and was effected through cytochrome oxidase.

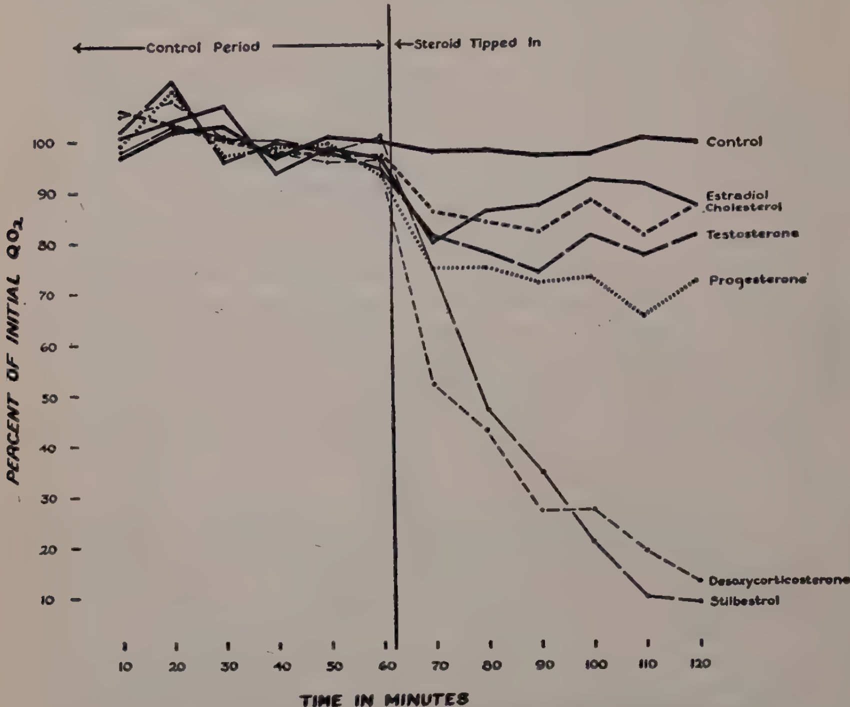

FIGURE 1. Effect of steroids on respiration of rat brain homogenates in glucose substrate.

TABLE 1

COMPOUNDS LISTED IN ORDER OF DESCENDING ANESTHETIC POTENCY. INHIBITION CALCULATED BY COMPARING Q_{O_2} AT THE END OF THE EXPERIMENTAL PERIOD WITH THE AVERAGE Q_{O_2} OF THE CONTROL PERIOD

Compounds	*No. of vessels*	*A.R.U.* in mg.*	*0.2 % glucose substrate*
			%
Desoxycorticosterone	12	1.0†	87
Progesterone	10	2.0	28
Testosterone	18	7.0	24
Stilbestrol	19	20.0	91
α-Estradiol	9	>20.0	14
Cholesterol	10	Inactive	14

* Anesthetic rat unit.
† Selye's value for desoxycorticosterone acetate.

Methylene blue did not, however, reverse the *steroid*-induced inhibition of the oxygen consumption of rat brain homogenates respiring in a glucose substrate in any case. Therefore, it is unlikely that this action is produced by an effect on cytochrome oxidase. Since methylene blue can act as a carrier between dehydrogenases and oxygen,[11] its failure to reverse the inhibition of glucose or pyruvate oxidation indicates a site of action pre-

ceding the flavoproteins. The cytochromes are probably not involved, since amounts of testosterone which inhibit glucose oxidation do not interfere with the oxidation of succinate. It has also been determined that steroids inhibit the respiration of rat brain in a pyruvate substrate.[16] It thus seems that the site of steroidal inhibition, demonstrable upon glucose

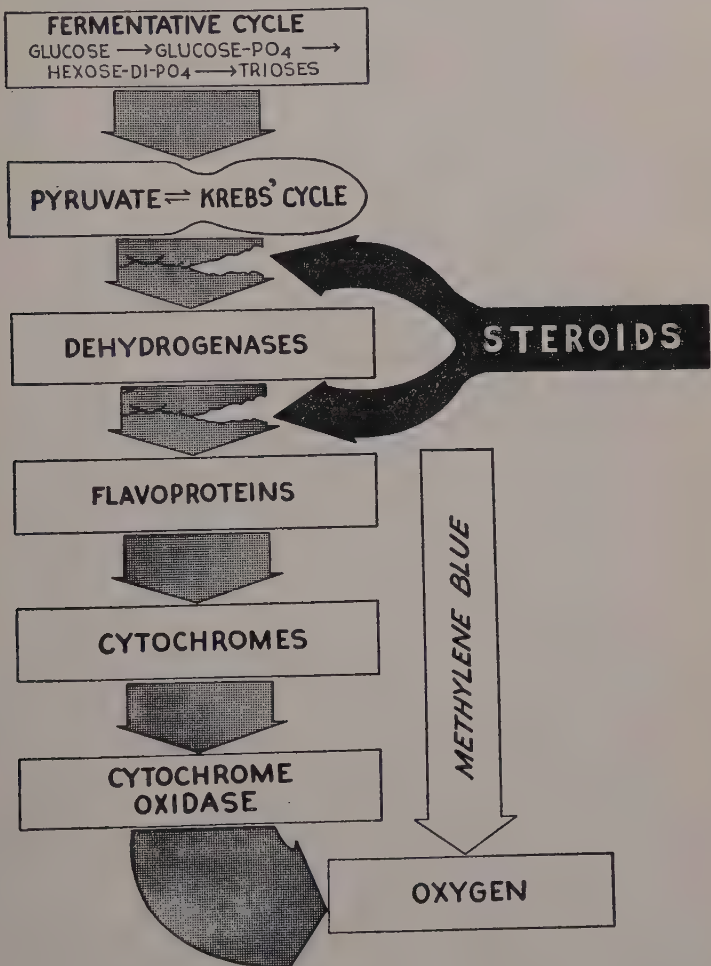

FIGURE 2.

or pyruvate and not reversed by methylene blue, lies at the level of the dehydrogenases, as indicated in FIGURE 2.

Since anesthetic agents are believed to produce their biological effects by inhibiting the utilization of carbohydrate at the cytochrome level,[2] it appears that steroids have a different mode of action than do the usual anesthetics.

C. CONCENTRATION-ACTION RELATIONSHIPS. At this point, we were concerned whether to pursue investigation upon specific isolated enzyme systems

or to study hormonal effects upon the total organism. Because of our particular interests and training, we felt more at home studying metabolism of the living organism than *in vitro* systems. Another point which helped our decision was the fact that relatively large amounts of steroids were used to produce inhibition of aerobic respiration. This is best exemplified in the studies of concentration-action relationships which were undertaken to determine whether the *in vitro* effects could be produced by physiologic concentrations of steroid. It was at first assumed that the low aqueous solubility of the compounds would cause any excess to act simply as a reservoir, and that the solution in the Warburg flask would be saturated or supersaturated as long as any excess was present. This assumption led to the use of large amounts of suspended steroid, as well as the selection of a tissue with a high lipid content dispersed as a cellular suspension, to increase surface area. Further study[12] was undertaken by determining the effect of varying concentrations of a highly potent steroid upon the oxygen consumption of a standard rat brain cell suspension. Since desoxycorticosterone (DC) produced greater inhibition than the other steroids studied, it was selected as a suitable compound for comparison of concentration-action relationships. Comparisons were also made using similar amounts of a water-soluble conjugate, desoxycorticosterone glucoside (DCG).

The experimental data for desoxycorticosterone are plotted in FIGURE 3. It will be observed that increasing amounts of steroid produced increasing degrees of inhibition; that increasing amounts of steroid (within the range of concentrations used) require increasingly longer periods of time to attain a maximum effect; and that maximal effect, once attained, is constant, with no increase or release of this respiratory inhibition within a period of two hours. As shown in FIGURE 4, the concentration-effect relationship is reasonably linear when the logarithm of the amount of steroid is plotted against the per cent inhibition. Desoxycorticosterone glucoside, although apparently somewhat less potent than the free steroid, also shows increasing effect with larger amounts in the range studied (shown with ringed dots).

Since the larger amounts of steroid required a longer period of time to produce a maximal inhibition, which was thereafter maintained at a constant level, it appeared that the steroid entirely penetrated or was adsorbed (or utilized) by the affected system *in vitro*. To test the validity of this conclusion, the preparations resulting from 60-minute incubation of the brain suspension with one, two, and four milligrams of desoxycorticosterone at 37.2°C were filtered, and the filtrates examined for steroid by the Zimmermann reaction and for effect on respiration of other brain suspensions. No steroid could be detected in the filtrate by either method, indicating complete fixation of the steroid by the system.

The amount of desoxycorticosterone necessary to produce minimally significant inhibition of aerobic respiration *in vitro* was in the neighborhood of 100 micrograms, a greater concentration than that which has been found necessary to affect other systems *in vitro* and probably very much greater than occurs in the living organism. Bartlett and MacKay[13] found that 30 micrograms of desoxycorticosterone reduced the amount of glycogen formed

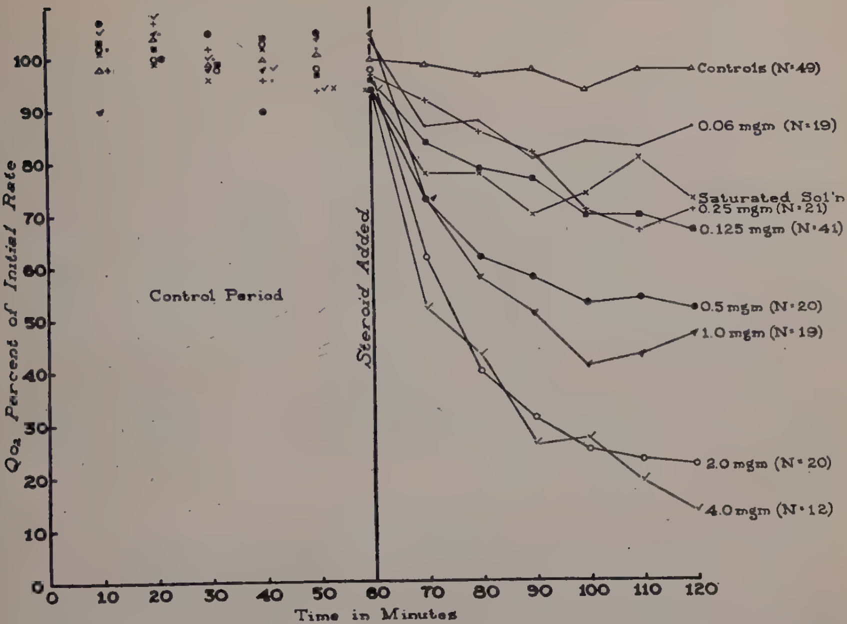

FIGURE 3. Effect of DC upon respiration of rat brain suspension *in vitro*. (Reprinted from EISENBERG, E., G.S. GORDAN, H. W. ELLIOTT, & J. TALBOT. 1949. Proc. Soc. Exper. Biol. & Med. **73:** 140.)

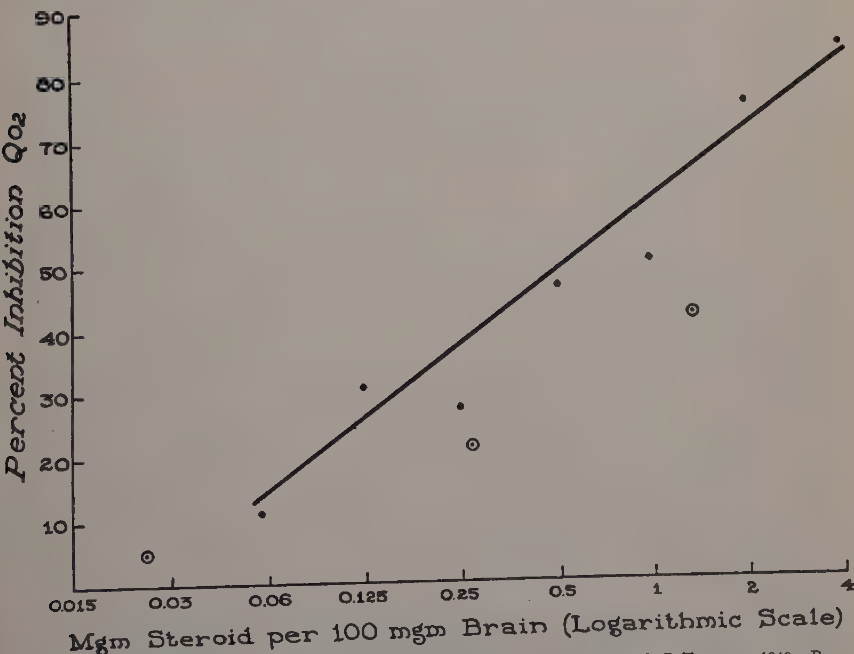

FIGURE 4. (Reprinted from EISENBERG, E., G. S. GORDAN, H. W. ELLIOTT, & J. TALBOT. 1949. Proc. Soc. Exper. Biol. & Med. **73:** 140.)

when skeletal muscle is incubated with glucose. It is thus seen that the amounts of steroid involved are of roughly the same order. Stimulation of oxygen consumption of the brain *in vitro* was not observed with any dose.

D. EFFECTS OF CASTRATION. *1. Respiration of the Brain of the Castrate Rat.* In an attempt to determine whether steroids are concerned with normal oxidative mechanisms, we studied the effects of castration upon the respiration of rat brain homogenates.[14]* Tissue respiration studies were performed upon three groups of animals: a group of normal males, a second group castrated at the age of 30 days, and a third group castrated at 30 days and afterward treated with one milligram of testosterone propionate per day subcutaneously. The rats were decapitated at 60 days of age and the rate of oxygen consumption of brain cellular homogenates was determined by the direct method of Warburg. When testosterone was added *in vitro*, two milligrams of a fine suspension were added after a 90-minute control period. Q_{O_2} was calculated as microliters of oxygen utilized per milligram of dry brain per hour. Wet/dry ratios were determined by the method of Crismon and Field.[15]

The experimental data are presented in TABLE 2. It will be observed that the rate of oxygen uptake by the brain of the castrated rat is 32 per cent higher than that of the normal rat ($p < 0.01$). The brain of the castrated rat treated with testosterone respires at a rate which is more nearly normal than that of the untreated castrate, although the rate is still slightly higher than normal ($p < 0.01$). While the *in vitro* oxygen consumption of the brain cellular suspension of the normal group falls off considerably over 180 minutes, the data indicate that the oxygen consumption of the brain from the castrate group and the testosterone-treated castrate group is maintained more near the initial level over this period. These differences between the normal group, on the one hand, and the castrate and testosterone-treated castrate groups, on the other, suggest that some factor other than testosterone may be lacking after gonadectomy performed upon immature rats.

In subsequent studies,[16] we have found that castration, when performed upon older or malnourished male rats, does not result in an elevation of the oxygen consumption of the brain. Thus age, degree of maturity, nutritional status, and possibly strain, are important factors in the production of this phenomenon.

(a) EFFECT OF TESTOSTERONE *IN VITRO*. The addition of testosterone *in vitro* further suppresses the oxygen uptake of all groups. The inhibition is far less in the castrates, however, than in either the treated castrates or the normal group. It may be fortuitous that the oxygen consumption of the brain of the untreated castrated rat to which testosterone has been added *in vitro* (5.3) is the same as that of the testosterone-treated castrated rat brain (5.4) at a comparable time. The rate of oxygen uptake by the brain of the treated castrated rat can be decreased to a still lower level

* Adrenalectomy was not deemed the most promising method of studying effects of steroidal deficiency, although corticoids were the most potent inhibitors of aerobic oxidation. Our reluctance to use this approach stems from the previous observation of Crismon and Field[15] that the altered Q_{O_2} of brain following adrenalectomy is merely a reflection of altered water content.

(2.6) by the further addition of testosterone *in vitro*. The variations in Q_{O_2} cannot be explained by differences in water content, since the moisture content of all groups is essentially the same.

(b) "BRAKING" ACTION OF TESTOSTERONE *IN VIVO*. Inasmuch as the potential enzymatic activity of certain tissues exceeds the actual activity found *in vivo*, the concept of the metabolic "brake" has been postulated.[17, 18] It appears that testosterone exerts such a "braking" action upon the oxidation of glucose by the brain of the immature rat, at least as measured *in vitro*. This is borne out by the high Q_{O_2} of the brain of the castrated male rat and partial prevention of the rise of Q_{O_2} by testosterone treatment *in vivo*. The further addition of testosterone *in vitro* is capable of restraining still further the oxygen consumption in normal rats and testosterone-treated

TABLE 2

RESPIRATION OF BRAIN CELL SUSPENSIONS FROM NORMAL, CASTRATE, AND TESTOSTERONE-TREATED CASTRATE MALE RATS: EFFECT OF TESTOSTERONE *in Vitro**

	Number of animals	*Mean Q_{O_2} first 90 min. $\mu l \pm \sigma m$†*	*Final Q_{O_2} 180 min. (no testosterone) $\mu l \pm \sigma m$*	*Final Q_{O_2} 180 min. (testosterone added at 90 min.) $\mu l \pm \sigma m$*	*Inhibition of Q_{O_2} produced by testosterone %*	*Wet/dry ratio*
Normal.....	12	6.5 ± 0.1 (35)‡	4.3 ± 0.1 (25)	1.5 ± 0.1 (10)	65	4.9
Castrate untreated....	16	8.6 ± 0.1 (72)	6.8 ± 0.1 (30)	5.3 ± 0.1 (42)	22	4.7
Castrate treated....	13	6.9 ± 0.1 (80)	5.4 ± 0.1 (28)	2.6 ± 0.1 (52)	52	4.7

* Reprinted from EISENBERG, E., G. S. GORDAN, & H. W. ELLIOTT. 1949. Science **109:** 337.
† σm — Standard deviation of the mean.
‡ Number of vessels in parentheses.

castrates. This was not found with untreated castrates. The brain of the castrated rat is less sensitive to the oxidation-inhibiting effect of testosterone *in vitro* than is the brain of the normal rat or that of the castrated rat treated with testosterone. A possible interpretation is that the intracellular concentration of the enzyme system through which testosterone exerts its inhibiting effect upon the oxygen uptake of brain is decreased after castration ("atrophy") and that this decrease can be prevented for the most part by the administration of testosterone.

2. Other Tissues. That the metabolism of the brain is, in many respects, unique, has been firmly established. Because of this, it is dangerous to apply analogies from data obtained with other tissues to the brain.

Our studies were therefore extended to other tissues in order to determine whether the effects of castration and the administration of testosterone *in vivo* and *in vitro* upon the respiration of brain homogenates were similarly produced elsewhere.[19] Tissues from three groups of male rats were studied: (1) a group castrated at 30 days of age; (2) a second group which was cas-

trated at the same age and injected subcutaneously with one milligram of testosterone propionate in sesame oil daily during the following 30 days; and (3) a third group of 60-day old normal males. All animals were killed by decapitation at 60 days of age. The respiration of slices of liver, diaphragm, and the levator ani muscle were studied by the direct method of Warburg. The respiration of liver and diaphragm was studied in two media: one in extracellular Ringer's (ER) without added substrate, and the other in extracellular Ringer's to which 0.2 per cent glucose had been added (EGR). When testosterone was added *in vitro*, it was placed in the side arm as 0.2 milliliters of an aqueous suspension containing two milligrams of finely powdered testosterone. In order to determine specificity, several carcinogenic phenanthrenes (3 methylcholanthrene, 1,2-benzanthracene, 1,2,5,6-dibenzanthracene, and 3,4-benzpyrene) were also added *in vitro*. These proved inert.

Striking differences in the effect of castration upon the oxygen consumption of rat brain and upon the oxygen consumption of rat liver, diaphragm, and levator ani muscle are indicated in TABLE 3. In contrast to brain, the oxygen uptake of liver, diaphragm, and levator ani is not increased by castration. The administration of testosterone to castrate animals *in vivo* further depressed the oxygen consumption of liver as measured *in vitro* but had little effect upon the respiration of either diaphragm or levator ani muscle. The oxygen consumption of the tissues studied was inhibited by testosterone *in vitro*, but the inhibitory influence was less pronounced with tissues from castrated rats. A similar insensitivity to the inhibitory effects of testosterone was also observed in the brain of the castrate rat. The four carcinogenic phenanthrenes added to cellular homogenates of rat brain *in vitro* had no effect on oxygen consumption.

Since liver slices from castrates respired at a lower than normal rate and since treatment of the castrate animal with testosterone *in vivo* further inhibited hepatic oxygen consumption, the participation of some other gonadal factor is again suggested. An explanation of the difference between the various tissues in response to castration and to the administration of testosterone *in vivo* is not apparent.

3. Specificity of the Restraining Action of Steroids in Vivo. The failure of the carcinogens to alter the oxygen consumption of rat brain cell suspensions, as well as the quantitative differences between the efficacy of the various steroids, indicates that the effect is not due merely to the steroidal nature of the molecule. An attempt was made to determine whether this effect was specific for testosterone or general among the hormonally active steroids.[20] Male rats, castrated at 30 days of age, were treated daily with one milligram of steroid subcutaneously per kilogram of body weight for the seven days preceding decapitation at the age of 60 days. The oxygen consumption of brain cell suspensions was determined by the direct method of Warburg, using glucose as a substrate.

The data obtained with various compounds are shown in TABLE 4. The brain of untreated castrates and estradiol dipropionate-treated animals demonstrated rapid rates of oxygen consumption. The administration of

TABLE 3

OXYGEN UPTAKE OF LIVER, DIAPHRAGM, AND LEVATOR ANI OF NORMAL, CASTRATE, AND TESTOSTERONE-TREATED CASTRATE RATS*

Tissue	Q_{O_2} *during first 90 min.*	Q_{O_2} *at end of 180 min.*	Q_{O_2} *at end of 180 min. Testosterone added at 90 min.*	*% of inhibition*
Liver				
Normal				
EGR	6.5 ± 0.2† (32)‡	5.3 ± 0.7 (8)	2.7 ± 0.1 (24)	49
ER	6.1 ± 0.2 (28)	4.7 ± 0.3 (8)	2.2 ± 0.1 (20)	53
Castrate				
EGR	5.2 ± 0.2 (34)	3.8 ± 0.6 (10)	3.0 ± 0.2 (24)	21
ER	5.7 ± 0.1 (28)	4.1 ± 0.2 (10)	3.0 ± 0.1 (18)	27
Castrate-treated				
EGR	4.5 ± 0.3 (33)	3.4 ± 0.5 (12)	1.9 ± 0.2 (21)	44
ER	4.8 ± 0.2 (34)	3.7 ± 0.4 (12)	1.7 ± 0.1 (23)	54
Diaphragm				
Normal				
EGR	6.3 ± 0.1 (30)	5.8 ± 0.5 (6)	3.2 ± 0.1 (24)	45
ER	6.3 ± 0.1 (28)	5.7 ± 0.4 (6)	3.1 ± 0.1 (22)	46
Castrate				
EGR	5.9 ± 0.1 (35)	5.6 ± 0.1 (12)	3.8 ± 0.1 (23)	32
ER	6.1 ± 0.1 (30)	5.2 ± 0.2 (8)	3.7 ± 0.1 (22)	29
Castrate-treated				
EGR	5.9 ± 0.1 (29)	5.4 ± 0.4 (8)	3.0 ± 0.1 (21)	44
ER	5.5 ± 0.1 (30)	5.0 ± 0.3 (8)	2.7 ± 0.1 (22)	46
Levator Ani				
Normal				
EGR	3.5 ± 0.4 (23)	2.5 ± 0.5 (5)	1.4 ± 0.1 (18)	44
Castrate				
EGR	3.4 ± 0.2 (14)	3.7 ± 0.3 (5)	2.1 ± 0.2 (9)	43
Castrate-treated				
EGR	3.2 ± 0.1 (23)	2.0 ± 0.1 (7)	1.1 ± 0.1 (16)	45

* Reprinted from EISENBERG, E., G. S. GORDAN, & H. W. ELLIOTT. 1949. Endocrinology 45: 113.
† Figures are the mean ± standard deviation of the mean.
‡ Figures in parentheses indicate the number of vessels.

TABLE 4

EFFECT OF STEROIDS *in Vivo* ON OXYGEN CONSUMPTION OF BRAIN OF CASTRATED MALE RATS*

Number of vessels	*Group*	Q_{O_2} *(µl/mg. dry weight/hr.)*
28	Intact rats	5.4 ± 0.1
26	Untreated castrates	7.3 ± 0.1
14	Estradiol dipropionate	6.5 ± 0.1
21	ACTH 1.0 mg. 4 times daily	5.8 ± 0.1
13	Methyl testosterone	5.6 ± 0.1
30	Testosterone propionate	5.5 ± 0.1
20	Desoxycorticosterone acetate	5.5 ± 0.1
44	Testosterone (unesterified)	5.1 ± 0.1
52	Progesterone	5.1 ± 0.1
14	Testosterone cyclopentylpropionate	5.1 ± 0.1
27	Ethinyl testosterone + $CHCl_3$	4.7 ± 0.1
28	Testosterone propionate + $CHCl_3$	4.5 ± 0.1

* Castrate age—30 days; treated with 0.1 mg. steroid per day, age 53–60 days; decapitated age—60 days.

adrenocorticotrophic hormone to castrate animals reduced the respiration to normal levels, indicating inhibitory activity of normal adrenal cortical steroids *in vivo*. Brains from castrates treated with unesterified testosterone propionate, methyl testosterone, testosterone cyclopentylpropionate, progesterone, or desoxycorticosterone acetate respired at normal rates. Ethinyl testosterone, because of its peculiar solubility, was administered in chloroform. As a control, testosterone propionate was also administered with chloroform separately injected. Since chloroform produced a great inhibition of aerobic respiration in the testosterone propionate-treated animals, it is not possible to determine to what degree ethinyl testosterone may have had an inhibitory effect. From these studies, it appears that steroids with the androstene nucleus, other than testosterone, are effective in restoring the "braking" action normally exerted by gonadal steroids upon cerebral metabolism, as measured *in vitro*.

II. EFFECT OF STEROIDS ON THE CEREBRAL METABOLISM OF MAN

The fact that steroidal effects upon brain metabolism determined under *in vitro* circumstances are not necessarily applicable to the intact organism made it desirable to pursue further investigations under completely *in vivo* conditions. Most species are not suitable for studies of brain metabolism *in vivo* because of the diffuse anastomoses between cerebral and extracerebral venous networks. In man, the monkey, and the rabbit, however, the cerebral vasculature is so arranged that mixed cerebral venous blood may be obtained from the readily accessible internal jugular bulb.[21] Blood specimens obtained from either the right or left jugular bulb are comparable and represent mixed venous drainage from the entire brain.[22, 23] Kety and Schmidt have demonstrated that blood from the internal jugular bulb is negligibly contaminated from extra-cerebral sources.[23] The discrete character of the venous drainage of the human brain, together with the relatively large blood volume of man, make this species the most suitable for studies of cerebral metabolism *in vivo*.

There is also a suitable method, the original nitrous oxide technique of Kety and Schmidt,[23, 24] for the determination of cerebral blood flow in man. An estimation of the rate of cerebral blood flow permits a computation of the rate of utilization or production by the brain of any substance which can be accurately analyzed in blood.

A. Previous Indications of Steroidal Activity on Human Cerebral Metabolism. Evidence of an *in vivo* action of steroidal hormones on cerebral functions in man may be inferred from many observations. Anesthesia has been reported to follow the intra-muscular injection of 11-hydroxyandrosterone in man.[25] Pincus and Hoagland[26–29] found that the administration of $\Delta 5$-pregnenolone to human subjects resulted in a significant raising of the threshold to fatigue in psychomotor tests. Euphoria, exhilaration, depression, well-being, and convulsions have been reported from the administration of various steroids in man.[28–35] We have been sufficiently impressed with the effects of steroids on the central nervous system in man to wonder

whether they may not mislead the physician into attributing well-being, especially if accompanied by anabolic activity, to a specific effect upon the underlying disease process.

The electrical activity of the cerebral cortex as recorded by the electroencephalograph may be altered or abnormal in various endocrine states. Physiological alterations in hormonal balance, such as those which occur during the menstrual cycle[36] and pregnancy,[37] are correlated with changes in the electroencephalogram. Electroencephalographic alterations have recently been demonstrated in patients with androgenic adrenal cortical hyperplasia,[38] a condition characterized by excessive production of abnormal steroids by the adrenal cortex.

Abnormally slow brain waves are found in adrenal cortical insufficiency (Addison's disease).[39–42] The electroencephalographic abnormalities disappear following treatment with various steroidal compounds, including whole adrenal cortical extract[39–41] and cortisone.[42] Adrenalectomized rats display a similar slowing of the brain waves which can be restored to normal by the administration of lipo-adrenal extract or Δ5-pregnenolone, but not by treatment with physiological saline or with small doses of desoxycorticosterone acetate.[43]

The anticonvulsive activity of desoxycorticosterone acetate administered to epileptic patients demonstrates that this steroid may exert objective effects upon the human brain *in vivo*. McQuarrie, Anderson, and Ziegler[44] and, more recently, Aird and Gordan[45] and Klein and Livingston[33] have reported a reduction in the frequency of convulsive episodes of epileptic patients receiving desoxycorticosterone acetate. The observation by Woodbury and Davenport,[46] that the electro-shock-seizure threshold is restored to normal levels in adrenalectomized rats and raised above normal levels in intact animals by desoxycorticosterone acetate, correlates well with the anticonvulsive action of this steroid in epileptic patients.

B.—EFFECT OF DESOXYCORTICOSTERONE GLUCOSIDE (DCG) ON HUMAN CEREBRAL METABOLISM *IN VIVO*. The rapidity with which cerebral effects may follow the administration of a steroid compound has been demonstrated. Aird and Gordan[45] have shown that epileptic patients who receive an intravenous injection of 50 milligrams of desoxycorticosterone glucoside (DCG), a potent water-soluble conjugate of desoxycorticosterone, show an immediate reduction of their electroencephalographic abnormalities (FIGURE 5). This effect is short lived. Within eight to ten minutes, the pre-injection electroencephalographic pattern reappears.

The combination of a potent water-soluble steroid with rapid cerebral activity and the recent development of a quantitative method for the estimation of cerebral blood flow and metabolism in man have made studies of steroidal effects upon human cerebral metabolism feasible.

1. Methods. Pilot studies were undertaken to determine the effect of desoxycorticosterone glucoside (DCG) upon the rate of cerebral blood flow and upon the rate of oxygen and sugar utilization and carbon dioxide production by the brain.[47] Cerebral blood flow (CBF) and cerebral metabolic rate (CMR) were estimated by the original nitrous oxide method of Kety

and Schmidt[23, 24] in a group of 15 human subjects consisting of seven without endocrine disease and eight with various endocrine disorders. The subjects received no medication for 24 or more hours before the study, and

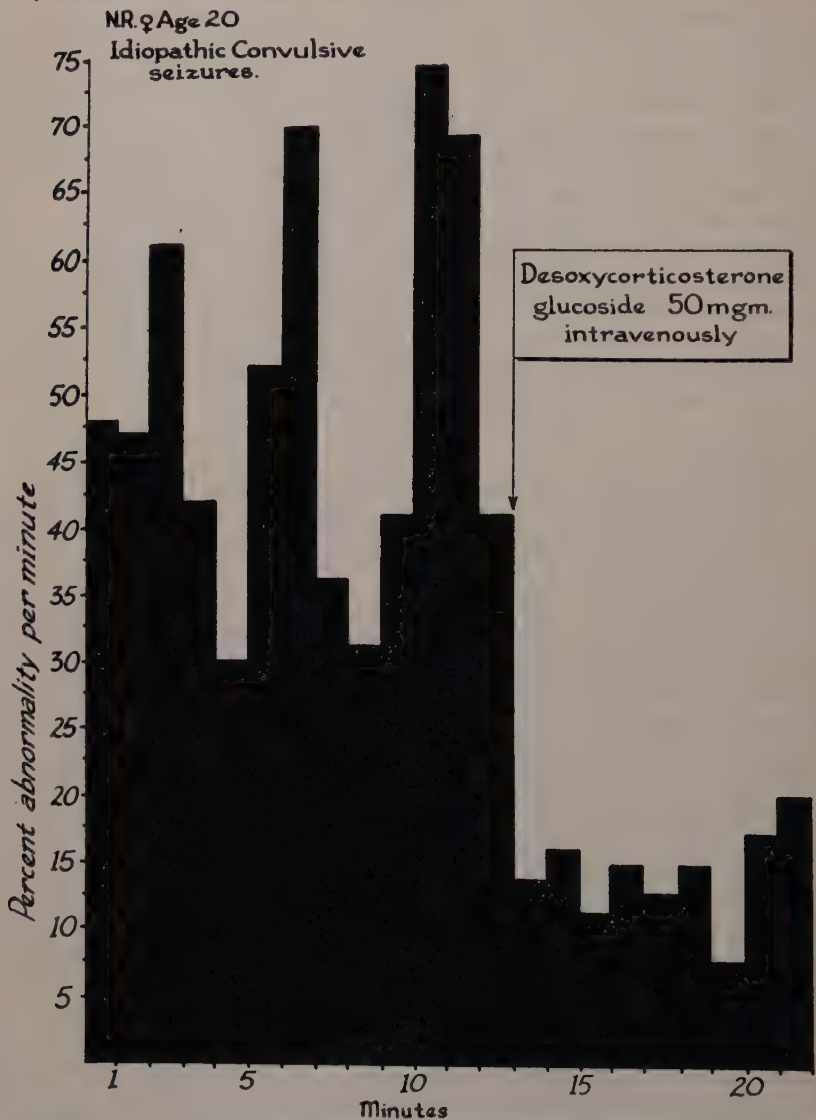

FIGURE 5. Effect of DCG on EEG in epilepsy. (Reprinted from AIRD, R. B. & G. S. GORDAN. 1951. J.A.M.A. 145: 715.)

hormonal medications had been withheld for the preceding two or more weeks.

With the fasting subject supine, needles were inserted into the jugular bulb and either the femoral or internal carotid artery and connected to

heparinized manifolds as described by Kety and Schmidt.[23] In order to avoid the effects of emotional stimuli, as stressed by Scheinberg and Stead,[48] no further procedures were undertaken until a steady state was achieved, as determined by stable cardiac rate and blood pressure. In 14 of the 15 patients, two determinations of the rate of cerebral blood flow were made consecutively. Twenty minutes were allowed to elapse after the control determination to permit expiration of nitrous oxide, and a second blood blank for nitrous oxide was obtained in each case. Prior to proceeding with the second cerebral blood flow determination, specimens for control glucose levels of arterial, cerebral venous, and, in seven cases, peripheral (antecubital) venous blood were obtained. All of these were drawn simultaneously at two-minute intervals until three separate sets of control specimens had been obtained.

Immediately after the third set of control samples for sugar determination were taken, 50 milligrams of DCG were injected intravenously, and a second determination of the cerebral blood flow was made without delay. Blood sugar values were determined on aliquots of the simultaneously drawn experimental specimens throughout the procedure for comparison with the previously drawn control specimens. Following completion of the second cerebral blood flow determination, two additional sets of specimens were obtained for analysis of sugar levels at three-minute intervals. When peripheral (antecubital) venous specimens were obtained, they were drawn at the same time as the arterial and cerebral venous specimens. During each cerebral blood flow determination, arterial and cerebral venous oxygen and carbon dioxide contents were determined upon separate specimens consisting of 5 ml. of blood drawn immediately before the first CBF sample and an equal amount drawn into the same syringe directly following the five-minute CBF specimen. Blood oxygen and carbon dioxide analyses were made in duplicate by the manometric technique of Orcutt and Waters,[49] as modified by Kety and Schmidt.[23] Total blood sugar content was determined by the iodometric titration method of Somogyi[50, 51] upon copper sulfate-sodium tungstate filtrates.[52] Blood sugar analyses were performed in duplicate and were found to check to amounts less than one milligram per cent. Yeast fermentation studies[53] disclosed that the copper sulfate-sodium tungstate filtrates of blood specimens taken before DCG was injected contained only negligible quantities of non-fermentable reducing substances.

Mean arterial blood pressure was obtained directly from the femoral or carotid artery by means of an air-damped mercury manometer connected to the arterial manifold. Cerebral oxygen consumption, cerebral sugar consumption or production, and cerebral carbon dioxide production were calculated from the rate of cerebral blood flow by the formulae of Kety and Schmidt.[23]

2. Carbohydrate Metabolism. As illustrated in FIGURES 6 and 7, striking and rapid changes in blood sugar levels occurred immediately after the administration of 50 milligrams of DCG. These figures also show the transitory nature of this phenomenon. Uniformly, the cerebral venous sugar

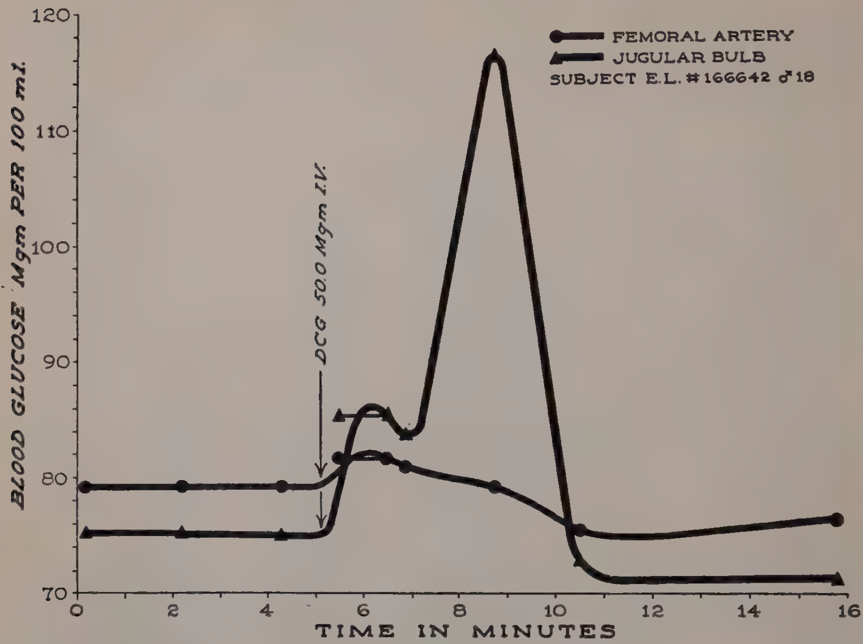

Figure 6. Cerebral glucose metabolism with desoxycorticosterone glucoside (DCG). (Reprinted from Bentinck, R. C., G. S. Gordan, J. E. Adams, L. H. Arnstein, & T. B. Leake. 1951. J. Clin. Invest. **30:** 200.)

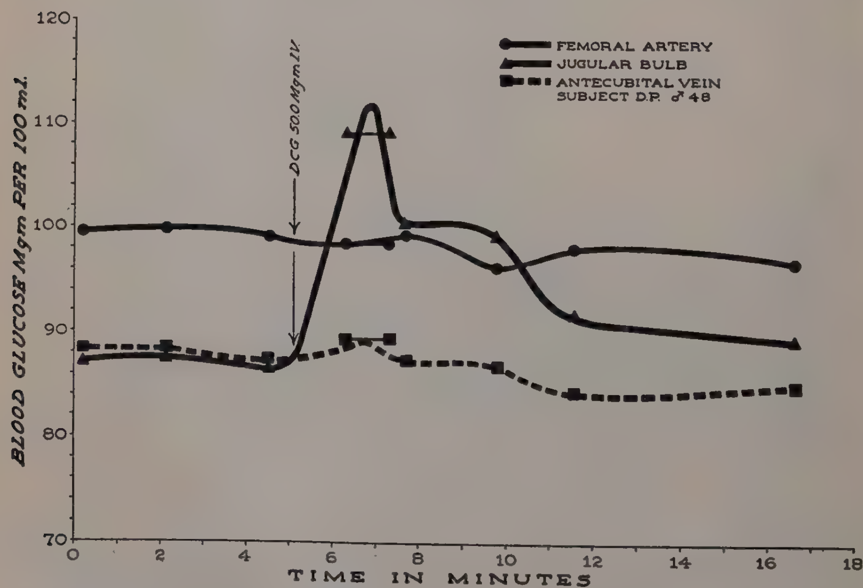

Figure 7. Cerebral glucose metabolism with desoxycorticosterone glucoside (DCG). (Reprinted from Bentinck, R. C., G. S. Gordan, J. E. Adams, L. H. Arnstein, & T. B. Leake. 1951. J. Clin. Invest. **30:** 200.)

concentration rose significantly—in eight instances above the arterial level—effecting, in 13 of the 15 cases, a reduction in the cerebral arterio-venous sugar difference. While the individual changes were inconsistent and not marked, a small, but statistically significant, rise occurred in both the mean arterial and the mean antecubital venous blood sugar concentrations following the administration of DCG. FIGURE 8 depicts the change in mean cerebral arterio-venous sugar difference produced by DCG. This demonstrates an increase of the mean cerebral venous sugar concentration above the mean arterial sugar concentration after the injection of DCG. An increase in the sugar concentration of the cerebral venous blood above the

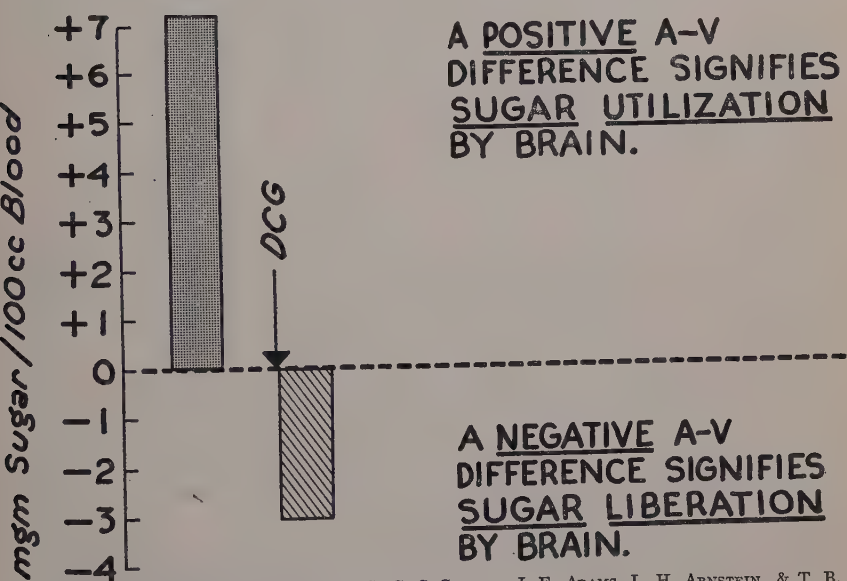

FIGURE 8. (Reprinted from BENTINCK, R. C., G. S. GORDAN, J. E. ADAMS, L. H. ARNSTEIN, & T. B. LEAKE. 1951. J. Clin. Invest. **30**: 200.)

level in arterial blood obtained simultaneously indicates frank release of sugar from the brain. In the absence of an augmented CBF, a decrease in the cerebral arterio-venous sugar difference may indicate release of sugar from the brain, or diminished cerebral utilization of sugar, or both. The release of sugar from the brain following the administration of DCG invalidates the use of the cerebral arterio-venous difference as an index of the effect of DCG upon the rate of oxidation of sugar by the brain.

The mean rates of cerebral oxygen consumption and carbon dioxide production obtained by aggregating this small group of subjects with divergent endocrine states were not significantly altered by DCG. The data suggested, however, that expansion of the individual groups might demonstrate differences in cerebral metabolism that could be correlated with endocrine status.

Studies have been continued to obtain statistically significant numbers

of subjects in each group, classified according to clinical endocrine status (normals, patients with hypopituitarism, adult male castrates, and patients with preadolescent testicular eunuchoidism). The procedure has been modified slightly: two injections of 50 milligrams of DCG, instead of one, were given directly into an antecubital vein in order to determine whether the labile carbohydrate source was exhausted by the first dose. Semi-integrated specimens for oxygen and carbon dioxide analyses, timed so as to reflect the results of each dose of DCG, were also taken, as well as additional specimens for sugar analysis.

(a) THE NATURE OF THE SUGAR RELEASED INTO CEREBRAL VENOUS BLOOD. Further investigations have also been undertaken to characterize the sugar released from the brain by DCG.[54] Values for total and non-fermentable reducing sugar were determined for each specimen of cerebral venous and arterial blood in eight subjects. Non-fermentable sugar was estimated as the residual reducing substance following the removal of glucose with bakers' yeast, according to the method of Somogyi.[53]

In three instances, the major portion of the increment in blood sugar was not glucose. While it reacted to the Molisch test as a saccharide, it was not fermented by bakers' yeast. This is presumptive evidence that the substance is galactose. Unfortunately, the total quantities obtained were insufficient for more definitive qualitative characterization. The formation of osazones or the determination of polarigraphic rotation was not feasible because of the small amounts involved.

FIGURE 9 illustrates two responses (liberation of sugar and increased arterio-venous difference, respectively) of the cerebral venous and arterial total blood sugar to separate injections of DCG in one subject. FIGURE 10 depicts the separation of the total reducing sugar values shown in FIGURE 9 into fermentable (glucose) and non-fermentable ("galactose") components. In this case, before and after the first injection of DCG, the arterial and cerebral venous blood were free of non-fermentable sugar. After the second injection of DCG, however, when sugar was released from the brain, non-fermentable reducing substance concurrently appeared in the blood. The cerebral venous level of "galactose" was higher than the arterial level, indicating that it was derived from the brain. Because the amount of "galactose" which appeared in the arterial blood was too great to be accounted for by the amount released from the brain, dispersed in the entire blood volume, a simultaneous release of non-fermentable sugar from extra-cerebral sources is indicated.

(b) THE SOURCE OF THE SUGAR RELEASED FROM HUMAN BRAIN *IN VIVO*. (*1*) *Methods*. An opportunity to investigate the cerebral precursors of the sugar liberated from the brain[54] was provided by the availability of biopsy specimens of human brain. Serial biopsies were obtained from 25 female psychotic patients during therapeutic prefrontal lobotomy performed under nitrous oxide anesthesia. Separate specimens of gray and of white matter were removed from adjacent areas of a prefrontal lobe immediately before and again two to three minutes after the intravenous administration of 100 milligrams of DCG. The glycogen content of each specimen was

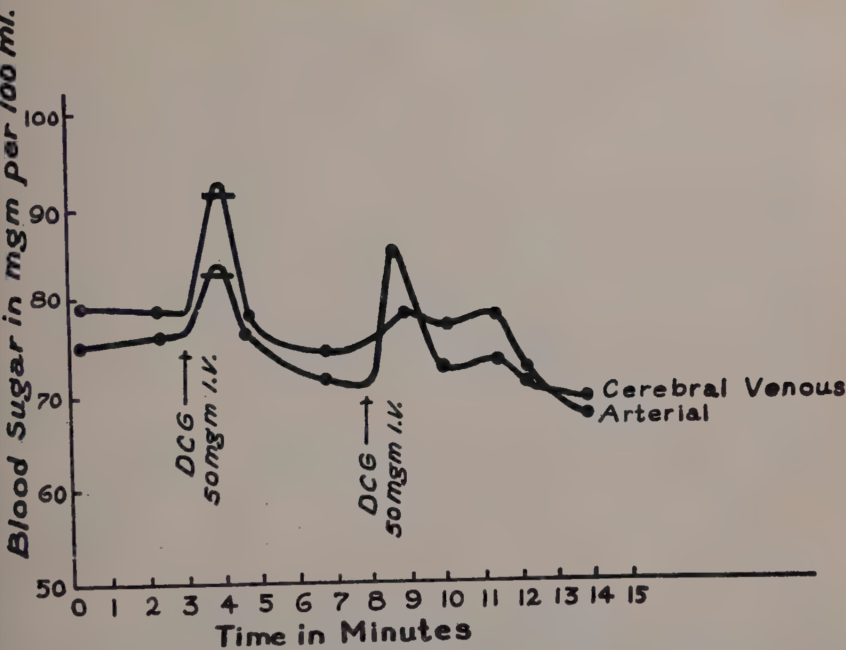

FIGURE 9. Pt. R. C. effect of desoxycorticosterone glucoside (DCG) on cerebral venous and arterial blood total sugar.

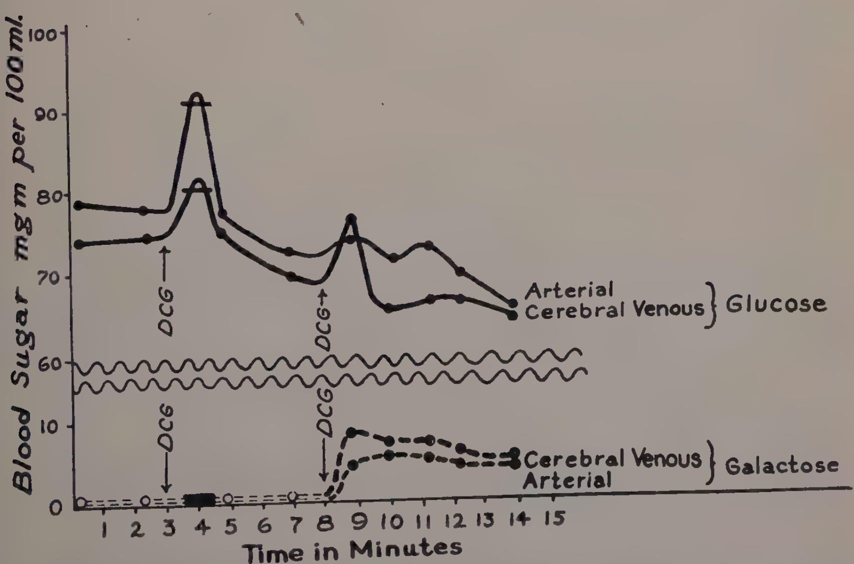

FIGURE 10. Pt.R.C.effect of desoxycorticosterone glucoside (DCG) on cerebral venous and arterial blood glucose and galactose.

determined by the method of Good, Kramer, and Somogyi,[55] modified according to Kerr,[56] for the separation of the cerebroside-containing fraction. Since a large store of carbohydrate is present in cerebrosides, the galactose content of this fraction was also determined. Hydrolysis was performed by the method of Kirk.[57] Non-carbohydrate reducing substance was removed by the addition of equal volumes of seven per cent copper sulfate and ten per cent sodium tungstate, and the remaining reducing substance present in the filtrate of each sample was analyzed in duplicate by the iodometric titration method of Somogyi.[50, 51] The glucose values were multiplied by the factor 0.927 to convert them to anhydrous glycogen.[58] All but negligible quantities of the glycogen fraction were fermentable by yeast after hydrolysis. Only negligible amounts of the galactose-containing cerebroside fraction were fermentable, indicating complete separation of the two fractions and the absence of non-carbohydrate reducing substances in the glycogen fraction.

TABLE 5

GLYCOGEN CONTENT OF CEREBRAL GRAY AND WHITE MATTER. AVERAGE AND RANGE OF VALUES FOR HUMAN SUBJECTS COMPARED WITH REPORTS FOR OTHER SPECIES*

Reference	*Species*	*Gray*		*White*		*Whole brain*	
		av.	*range*	*av.*	*range*	*av.*	*range*
Bentinck *et al.*	human	86	31–126	82	20–138		
Kerr & Ghantus	dog					98	77–150
Kerr & Ghantus	cat					86	77–101
Kerr & Ghantus	rabbit					82	70– 99
Chesler & Himwich	dog (adult)	73	45–108 (cortex)				
Chesler & Himwich	cat (adult)	68	42–120 (cortex)				
Klein & Olsen	cat					73	43–108
Chance	mouse	7.5 (cortex)					

* Mg. per 100 gms. of wet tissue.

(2) *Effect of DCG on the glycogen content of human brain.* TABLE 5 shows the glycogen content of human cerebral gray and white matter, average and range of values, as obtained by us, and also the values reported for other species. Our values for human subjects are in good agreement with those found by Kerr and Ghantus[59] for dogs, cats, and rabbits, slightly higher than those reported by Chesler and Himwich[60] for adult dogs and cats and by Klein and Olsen[61] for cats, and considerably greater than those reported by Chance and Yaxley[62] and by Chance[63] for mice.

The average galactose content of the cerebroside fraction of human cerebral gray and white matter before the administration of DCG is shown in TABLE 6. Assuming the theoretical galactose content of the cerebrosides to be 22 per cent, these values are considerably lower than those reported by Johnson, McNabb, and Rossiter[64] for autopsy specimens of normal adult human brain.

In order to determine the reproducibility of the glycogen and galactose values obtained from adjacent areas of a prefrontal lobe, the entire procedure, except that DCG was not administered, was followed in five cases. As indicated in TABLE 7, the glycogen and galactose contents of cerebral gray and white matter from the same subject obtained at intervals of two to three minutes showed little or no change.

TABLE 8 shows the glycogen content of the cerebral white and gray matter before and after the administration of DCG. Although the mean of all values was not significantly altered by DCG, certain patients showed increases or decreases well outside the apparent error of the method.

TABLE 6

GALACTOSE CONTENT OF CEREBRAL GRAY AND WHITE MATTER. AVERAGE AND RANGE OF VALUES FOR 25 PSYCHOTIC PATIENTS*

Gray		*White*	
Av.	*Range*	*Av.*	*Range*
55	11–187	229	64–329

* Mg. per 100 gms. of wet tissue.

TABLE 7

GLYCOGEN AND GALACTOSE CONTENT OF SERIAL SPECIMENS OF CEREBRAL GRAY MATTER AND WHITE MATTER FROM PSYCHOTIC PATIENTS*

Pt.	*Glycogen*				*Galactose*			
	Gray		*White*		*Gray*		*White*	
	Spec. 1	*Spec. 2*	*Spec. 1*	*Spec. 2*	*Spec. 1*	*Spec. 2*	*Spec. 1*	*Spec. 2*
J. J.	80	73	94	97	56	74	329	326
C. R.	56	55	103	99	80	74	265	252
M. R.	45	44	66	73	11	22	202	229
B. C.	120	106	137	147	29	32	310	336
A. B.	118	114	138	131				

* Mg. per 100 gm. of wet tissue.

(3) *Effect of DCG on the cerebroside-galactose content of human brain.* The galactose content of cerebral gray and white matter before and after the administration of DCG is similarly depicted in TABLE 9. Again, while the mean of all values was not significantly changed by DCG, marked individual exceptions are apparent. The decrease of the galactose content of the white matter was greater than the apparent error of the method in 3 of 10 subjects. There was no apparent correlation between the response of the glycogen or galactose content of the brain to the clinical status of these psychotic patients or to the type, frequency, or duration of previous therapeutic measures.

The individual variability in the response of the glycogen and galactose stores of the brain to the administration of DCG is consistent with the variable effects produced by DCG upon the concentration of sugar in the

TABLE 8

THE EFFECT OF DESOXYCORTICOSTERONE GLUCOSIDE (DCG) ON GLYCOGEN CONTENT OF HUMAN CEREBRAL GRAY AND WHITE MATTER*

No.	Patient	Gray matter		White matter	
		Control	DCG	Control	DCG
1	R. H.	42	21	20	26
2	E. M.	45	39	20	171
3	V. P.	100	104	88	75
4	R. A.	87	70	63	64
5	B. B.	156	140	128	158
6	C. S.	93	114	101	119
7	V. B.	107	86	76	
8	D. C.	99	83	44	71
9	B. L.	126	127	76	33
10	A. M.	125	119	86	126
11	V. H.	56	92	60	103
12	I. H.	38	30	40	28
13	M. K.	100	107	78	85
14	G. R.	115	125	109	88
15	G. O.	113		129	103
16	G. I.	57	47	74	79
17	B. H.	48	162	62	94
18	E. D.	31	19		
19	R. A.	106	64		
20	M. P.	95	54		
Mean of all observations..		87	84	74	89
Standard deviation of the mean................		8.0	10.0	8.0	10.1

* Mg. per 100 gm. of wet tissue.

TABLE 9

THE EFFECT OF DESOXYCORTICOSTERONE GLUCOSIDE (DCG) ON THE GALACTOSE CONTENT OF HUMAN CEREBRAL GRAY AND WHITE MATTER*

No.	Patient	Gray matter		White matter	
		Control	DCG	Control	DCG
1	B. B.	187	160	293	19
2	C. S.	89	90	126	96
3	D. C.	49	83	190	230
4	B. L.	72	61	173	128
5	A. M.	30	115	191	205
6	V. H.	40	37	161	125
7	I. H.	26	31	290	343
8	M. K.	26	119	64	119
9	G. R.	40	53	323	324
10	G. O.	38		282	282
Mean of all observations..		60	83	209	187
Standard deviation of the mean................		16.3	15.4	28	36

* Mg. per 100 gm. of wet tissue.

cerebral venous and arterial blood, upon the cerebral A-V blood sugar difference, and upon the nature of the carbohydrate found in the blood. The mean levels of glycogen and galactose in the cerebral gray and white matter before and after the administration of DCG do not differ significantly, but increases or decreases greater than the apparent error of the method occur in individual instances. These changes are in keeping with the alterations in the A-V blood sugar differences.

The data indicate that changes in the cerebral arterio-venous glucose difference produced by DCG may be paralleled by inverse changes in the glycogen stores of the brain. It thus appears that cerebral glycogen may be mobilized by DCG in some instances. The lability of cerebral glycogen *in vivo* has been established. Chesler and Himwich[65] found a significant decrease in the glycogen content of the brains of dogs during insulin-produced convulsions. This observation has been confirmed by Olsen and Klein.[66] These authors also reported that convulsive activity induced by electric shock or by drugs is associated with decreased cerebral glycogen content.[61] The glycogen content of the brain has also been observed to decrease during extreme hypoxia in dogs.[67] Species variability of response may be suggested by the report of Chance[63] that an increase in the glycogen content of mouse brain occurred during convulsions induced by analeptic agents and by insulin. An influence of adrenal cortical steroids upon the glycogen of brain is indicated by the observations of Abood and Kocsis.[68] They found that the glycogen content of rat brain was reduced after hypophysectomy and that normal levels could be restored by treatment of the hypophysectomized animals with ACTH.

The ability of desoxycorticosterone to produce effects upon glycogen in various tissues has been demonstrated *in vitro* and *in vivo*. Verzar and Wenner[69] found desoxycorticosterone to be glycogenolytic *in vitro*. Verzar and Wang[70] demonstrated increases or decreases of glycogen in rat liver and muscle *in vivo*, depending upon the amount of desoxycorticosterone administered. Decreases of glycogen content were not found unless doses sufficiently large to produce a narcosis-like state were given. Wiesener[71] observed that small doses of desoxycorticosterone *in vivo* increased glycogenolysis, while higher doses decreased glycogenolysis of diaphragm and liver slices from mice. Chiu and Needham[72] observed that desoxycorticosterone is glycogenetic under slightly different *in vitro* conditions. This apparently dual potentiality is in accord with our observations.

Since glycogenolysis in human brain following DCG occurs irregularly, only limited speculation on this point is in order. It is of interest that desoxycorticosterone can produce glycogenolysis—and also glycogenesis—under slightly different conditions. Attempts to correlate these divergent observations with intracellular concentrations of sodium, potassium, or phosphate have produced controversial results.[44, 46, 73–77]

Of greater interest to us is the release of non-fermentable sugar ("galactose") into the cerebral venous blood and a decrease in cerebroside-galactose concentration of the brain in about one-third of the human subjects. Thus far, we have not had the opportunity to study the effect of DCG upon the

CBF, the blood sugars, and the glycogen and galactose contents of the brain in the same individual. It is recognized that such a balance study is critical to establish this point, particularly since, as indicated in TABLE 9, the cerebroside-galactose sometimes increased. The possibility that the "galactose" found in the cerebral venous blood may be derived, in part, from glycogen should be considered. There is some evidence that glycogen may contain galactose, for Stetten[78] has shown that galactose may enter glycogen without mobilization of the C-4 hydrogen. Our data suggest, however, that DCG may cause liberation of galactose from cerebrosides. An influence of steroids upon the cerebroside content of the brain has previously been demonstrated by Weil,[79] who found that smaller amounts of galacto-lipids were present in the brains of male than in female rats, and that this fraction was reduced by castration of either sex. These observations suggest that the brain cerebrosides are not merely rigid structural components but are potentially mobilizable by steroids. The mechanism of this phenomenon is not apparent.

C. CORRELATION OF ENDOCRINE STATUS AND CEREBRAL METABOLISM. *1. Clinical Material.* We have now measured cerebral metabolic rates (CMR_{O_2}), cerebral utilization of glucose, and the response to DCG in 37 human subjects.[80] These patients have been grouped according to clinical endocrine status. None of the patients were debilitated at the time of the study.

Early in the course of this investigation, there appeared to be differences between the cerebral metabolism of normal and of hyposteroidal subjects. We therefore set out to expand the groups in order to determine whether the apparent differences are statistically valid. We are still in the process of expanding the individual groups, but the data already speak for certain correlations.

The normal group was composed of 10 subjects, all in apparently normal hormonal status. A second group of nine patients who had miscellaneous non-endocrine cerebral diseases (cerebral vaso-spastic conditions, vascular thromboses, vascular anomalies, or convulsive states) was also studied. The third group was made up of nine patients with hypopituitarism. Hypophyseal eunuchoids were grouped with patients with frank hypopituitarism, rather than with the primary testicular eunuchoids. It will be apparent that this grouping proves most satisfactory from the standpoint of correlation and is in keeping with Albright's[81] contention that hypogonadotrophic eunuchoidism is actually a form of "panhypopituitarism." Our data in regard to the excretion of 17-ketosteroids and tests of adrenal cortical function, as well as the cerebral metabolic rate and behavior, confirm Albright's view. Two of these hypophyseal eunuchoids also had pituitary tumors.

The six patients with preadolescent testicular eunuchoidism are separated from the three adult males who had been castrated for prostatic carcinoma. This distinction was necessitated because: (1) earlier studies on animals had indicated that castration of immature rats produced different effects upon cerebral metabolism than when castration was performed upon mature

animals;[16] (2) the deficiency of gonadal androgens was probably not absolute in the preadolescent testicular eunuchoids (as it is after surgical castration); (3) the adult male castrates had been treated with stilbestrol until two weeks preceding the study; and (4) studies on animals had suggested that gonadal principals other than androgens may be factors in cerebral metabolism.[14]

Despite our interest in the effect of corticoids upon cerebral metabolism, we have thus far lacked the courage to perform studies upon patients with Addison's disease. The tenuous metabolic balance of such patients precludes withdrawal of supportive therapy for a sufficient period of time to obtain data which could be considered as representative of a basal state.

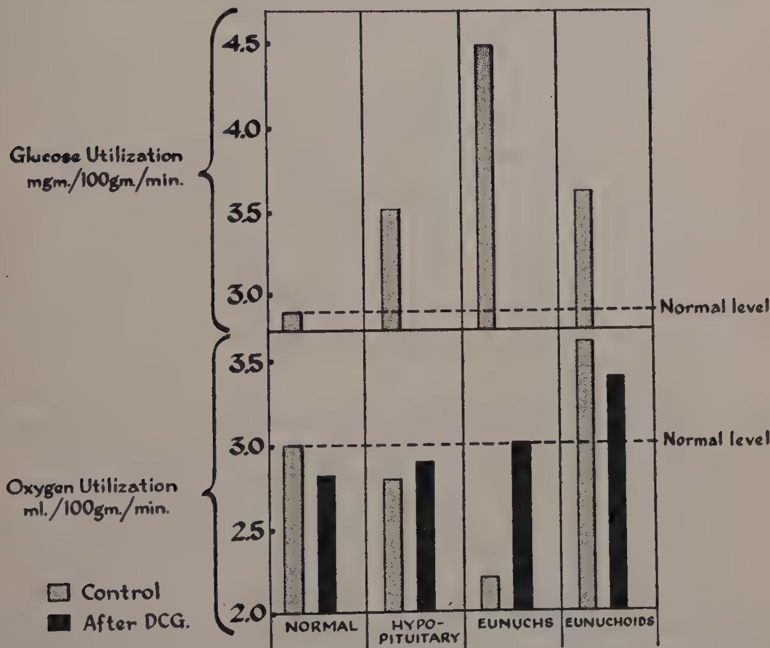

FIGURE 11. Cerebral utilization of glucose and oxygen and effect of DCG on CMR_{O_2} in various endocrine deficiencies.

Studies[15] have also indicated that abnormalities of the aerobic respiration of brain slices from adrenalectomized animals are, in essence, the result of dehydration. Perhaps future methods will make studies of cerebral metabolism in Addisonian subjects feasible and establish a metabolic basis for the known aberrations of cerebral electrical activity characteristic of this condition.

(a) CEREBRAL METABOLIC RATES. FIGURE 11 shows the mean rates of cerebral oxygen consumption (CMR_{O_2}) of the normal subjects and those with various endocrine deficiencies. Our values for the CMR_{O_2} of the normal group agree with previously reported figures.[23, 48, 82]

The reduced metabolism of patients with pituitary deficiency is reflected in the brain by a tendency to a sluggish cerebral metabolic rate. It is tempting to ascribe this reduction in the rate of cerebral oxygen consump-

tion to the deficiency of thyroid secretion which is known to be present, since a lower than normal rate of oxygen consumption by the brain has been demonstrated in human hypothyroid subjects.[83, 84] An extra-thyroidal factor must also be present, however, since the reduction in cerebral metabolic rate of pituitary deficiency is not only shared by, but is most pronounced in, the adult male castrates who presumably have no deficiency of thyroid hormone. The observation, that thyroxine produces a smaller increase in the oxygen consumption of adrenalectomized rats than in intact normal animals,[85] would also suggest the participation of a factor other than thyroid in cerebral oxidative processes.

The cerebral oxygen consumption of the adult male castrates is reduced to an even greater degree than in the patients with hypopituitarism. (It must be recalled that these castrate men had received treatment with stilbestrol for carcinoma of the prostate until two weeks before the study was made.) In the patients with preadolescent testicular eunuchoidism, the rate of cerebral oxygen utilization is somewhat greater than normal.

(b) RESPONSE TO DCG. The effect of DCG upon the mean rate of cerebral oxygen consumption of the various groups of subjects is shown in the same graph (FIGURE 11). It will be observed that the CMR_{O_2} of normal subjects tended to decrease after DCG. The CMR_{O_2} of the patients with hypopituitarism failed to decrease after DCG, and even increased very slightly. We have tried a similar experiment *in vitro* to determine whether DC would increase the rate of oxygen consumption of brain homogenates from hypophysectomized rats. The results of this study were in no way different from those obtained when DC was added to normal rat brain *in vitro*, *i.e.*, inhibition, not stimulation, was produced.

In both the testicular eunuchoids and the adult male castrates, an increased CMR_{O_2} resulted after the administration of DCG. It will be noted that in all groups the CMR_{O_2} was altered towards the normal level after DCG.

As indicated in TABLE 10, there was no apparent correlation between the clinical endocrine state and the frequency with which sugar was liberated from the brain by DCG.

(c) CARBOHYDRATE METABOLISM. The mean rates of cerebral glucose consumption before the administration of DCG are also shown for the various groups in FIGURE 11. Our values for all groups, except the adult male castrates, are somewhat lower than those previously reported from other laboratories.[82, 87, 88] The lower value seems to us the more accurate, since the Somogyi method, which was employed by us, excludes non-carbohydrate reducing substances. *It will be observed that the mean rates of cerebral glucose consumption are higher than normal in all groups with a clinical deficiency of one or more steroid hormones.* In these subjects, the steroidal "brake" on the utilization of glucose is clearly released.

We have not included data on the effect of DCG upon the rate of cerebral consumption of glucose. The likelihood that changes observed in this figure after the administration of DCG are in part due to the release of sugar from the brain invalidates any interpretations referable to the effect of DCG upon cerebral glucose consumption.

In one patient, cerebral metabolic studies* were conducted before, and again six weeks after, surgical hypophysectomy performed as experimental therapy for melanosarcomatosis. At the time of the second determination, the patient demonstrated clinical and laboratory evidence of full-blown pituitary insufficiency. It will be observed in TABLE 11 that hypophysectomy resulted in a decreased rate of oxygen consumption and an increased rate of glucose utilization by the brain. It can also be seen that the administration of DCG before hypophysectomy tended to decrease the CMR_{O_2}, while, paradoxically, it tended to increase the CMR_{O_2} after hypophysectomy. In this one case, where the control state could be compared with the *a*-pituitary state, we find confirmation for the changes in cerebral metabolism, noted to a lesser degree in the *hypo*pituitary patients.

TABLE 10

SUGAR LIBERATED FROM BRAIN IN RESPONSE TO DESOXYCORTICOSTERONE GLUCOSIDE (DCG)

Normal adults	4/9
Preadolescent testicular eunuchoids	3/6
Adult male castrates	1/3
Patients with hypopituitarism	4/9

TABLE 11

EFFECT OF SURGICAL HYPOPHYSECTOMY ON THE CEREBRAL UTILIZATION OF OXYGEN AND GLUCOSE (ONE PATIENT)

Before hypophysectomy			*After hypophysectomy*		
CMR_{O_2} (ml./100 gm. of brain/min.)		*$CMR_{glucose}$ (mg./100 gm. of brain/min.)*	*CMR_{O_2}*		*$CMR_{glucose}$*
Control	*After DCG*	*Control*	*Control*	*After DCG*	*Control*
3.5	2.9	1.7	2.6	2.8	5.0

TABLE 12

CEREBRAL A-V GLUCOSE:OXYGEN RATIOS (MG/ML.)

	Cerebral A-V glucose, oxygen ratio
Theoretical	1.34
Normal subjects	0.97
Preadolescent testicular eunuchoids	0.99
Adult male castrates	1.71
Patients with hypopituitarism	1.26

TABLE 12 shows the mean cerebral A-V glucose:oxygen ratios of the diverse groups before the administration of DCG. We obtained a ratio of 0.97 mg./ml. for the ortho-steroidal subjects. Gibbs *et al.*[89] reported a mean A-V glucose:oxygen ratio of 1.62 mg./ml. for schizophrenic and epileptic patients, and Scheinberg and Stead[48] reported a ratio of 1.67

* Studied through the courtesy of Dr. M. B. Shimkin of the Laboratory of Experimental Oncology, and Dr. E. B. Boldrey of the Division of Neurological Surgery, University of California School of Medicine

mg./ml. for normal subjects. The discrepancy between our figures and those of Gibbs, *et al.* and of Scheinberg and Stead is probably due to differences in cerebral A-V glucose values, since our cerebral A-V oxygen differences are comparable to theirs.

The theoretical cerebral A-V glucose:oxygen ratio of 1.34 is anticipated from the chemical equation of the complete breakdown of glucose to carbon dioxide and water. On the assumption that the R.Q. values of about one which have been obtained for brain indicate that all the glucose removed from the blood by the brain is completely oxidized to carbon dioxide and water, and that glucose is the only substrate involved in cerebral metabolism *in vivo*, the glucose:oxygen ratio of 1.34 has been accepted as theoretically applicable to brain.

Although there seems little doubt that the R.Q. of cerebral cortex is about one *in vitro*,[90] Himwich and Fazekas[91] reported values between 0.89 and 0.99 for different parts of rat brain. The respiratory quotients of our groups of subjects averaged from 0.90 to 0.97, which again suggests that the complete oxidation of glucose to carbon dioxide and water does not fully explain the energy metabolism of the entire brain *in vivo*. It is also entirely possible that mechanisms other than the complete oxidation of glucose may fortuitously give an R.Q. of one; for example, the oxidation of acetoacetate which, according to some evidence,[92, 93] may occur in brain.

A cerebral A-V glucose:oxygen ratio of less than 1.34 indicates either storage of carbohydrate or the participation of non-glucose substrates in cerebral oxidative processes, while a ratio greater than 1.34 denotes the partial diversion of glucose into other metabolic usage. Assuming that our studies reflect basal conditions, storage is necessarily excluded, for otherwise the brain would eventually be solid carbohydrate.

As indicated in TABLE 12, the normal group and the patients with preadolescent testicular eunuchoidism had similar mean cerebral A-V glucose:oxygen ratios of 0.97 and 0.99, respectively. The patients with hypopituitarism had a mean ratio of 1.26, which closely approximates the theoretical figure derived from the complete oxidation of glucose. On the other hand, a mean cerebral A-V glucose:oxygen ratio of 1.71 was obtained for the adult male castrates. The difference between the various groups strongly suggests that clinical endocrine status is related to human cerebral energy metabolism *in vivo*.

2. Discussion. The mechanism underlying the differences in the rate of cerebral oxygen and glucose utilization and the cerebral A-V glucose:oxygen ratio between the various groups of subjects may only be conjectured. There is relatively little available information pertaining to the effect of steroids upon the intermediary metabolism of brain. The dangers of applying data obtained by studies of isolated systems *in vitro* to the metabolism of intact brain *in vivo*, and of drawing analogies between brain and other tissues, are emphasized by our data. We feel, however, that certain speculations are justified, since they may suggest points of departure for subsequent studies.

A speculative scheme of cerebral intermediary metabolism which would

explain our data for normal subjects is shown in FIGURE 12. Some of the reactions which may be influenced by steroids are also indicated. Steroidal inhibition of the hexokinase reaction has been demonstrated.[94] Release of steroidal inhibition at this point would be reflected in an increased utilization of glucose as determined from the cerebral A-V glucose difference. The "glucose-6-phosphate shunt" is also shown, although its function need not be postulated to explain our data for normal subjects. A steroidal influence upon the Embden-Meyerhof cycle has been reported by Kun[95] and by Kun and McCurley.[96] Their evidence indicates that steroids activate the formation of phosphoenolpyruvate from 3-phosphoglycerate. This may be a factor in the preferential formation of triose by way of the Emb-

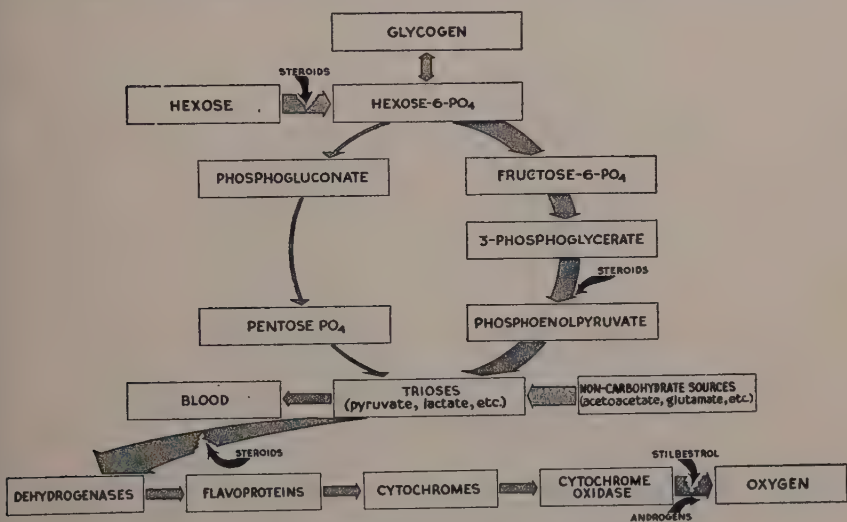

FIGURE 12. Normal brain.

den-Meyerhof cycle in normal subjects, as indicated by heavy arrows in FIGURE 12.

After the formation of triose, represented by pyruvate, other factors become active. Small amounts of pyruvate[97] and of lactate[88] are normally released from the brain into the cerebral venous blood, as indicated in the diagram. As shown, other substances may also enter the "main line of biological oxidation" at this level.

A continued cerebral A-V glucose:oxygen ratio of less than 1.34, as found in our normal subjects, would require the oxidation of some non-carbohydrate substrates. There is evidence that acetoacetate,[92, 93] for example, can be oxidized by brain. The participation of a substrate such as acetoacetate in normal cerebral oxidative metabolism *in vivo* would explain the greater amount of oxygen consumed than was required by the quantity of glucose which was removed from the blood.

FIGURE 13 depicts a speculative modification of the "main line of bio-

logical oxidation" which would explain our data for the patients with hypopituitarism and the adult male castrates. Both of these groups displayed a tendency to an increased cerebral glucose consumption, decreased oxygen consumption, and an increased cerebral A-V glucose:oxygen ratio as compared to the normals. This trend was most pronounced in the adult male castrates.

The increased glucose consumption by the brain of hypopituitary subjects is in accord with evidence indicating increased hexokinase activity and increased phosphorylation of glucose by deprivation of one or more steroidal hormones *in vivo*.[98–100] Since an increased cerebral consumption of glucose also occurred in the adult male castrates, who had no evidence of anterior hypophyseal insufficiency, as well as in the patients with hy-

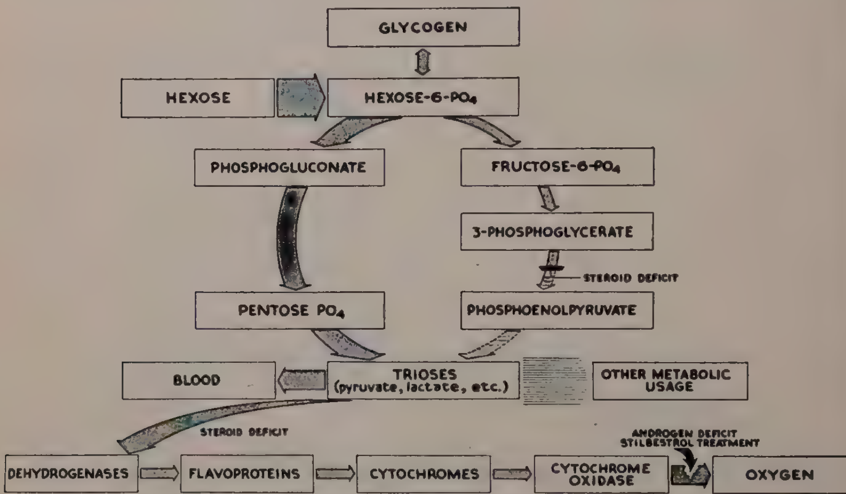

FIGURE 13. Brain in hypopituitarism and adult male castrates.

popituitarism, the restraining influence is probably mediated through the gonads, adrenals, or both.

As these patients were in a resting state when the studies were performed, it does not seem likely that the additional glucose-6-phosphate thus formed would be synthesized into glycogen. If Kun's observations[95, 96] may be extended to man, the Embden-Meyerhof cycle would be inhibited at 3-phosphoglycerate by a steroidal deficiency. There is evidence, however, that glucose can be oxidized by the brain through pathways other than those initiated in the Embden-Meyerhof scheme.[101–103] The formation of phosphogluconate from glucose-6-phosphate in the presence of an inhibitor of the anaerobic cycle has been demonstrated,[104] and the aerobic formation of pentose-phosphate from phosphogluconate has been demonstrated in cerebral tissue.[105] Accordingly, as indicated in FIGURE 13, triose formation might proceed by way of the "glucose-6-phosphate shunt" in the patients with hypopituitarism and in the adult male castrates. Since this path-

way involves oxidative decarboxylation, less pyruvate would be formed than by the Embden-Meyerhof cycle.

The hypopituitary patients and the adult castrates had a lower rate of cerebral oxygen consumption and a higher cerebral A-V glucose:oxygen ratio than the normals. The cerebral A-V glucose:oxygen ratio of 1.26 for the hypopituitary subjects suggests that all the oxygen was being used for the complete breakdown of glucose alone and that non-carbohydrate substrates were not being utilized. The cerebral oxygen consumption, however, was *less* than would be required for the complete oxidation of the amounts of glucose utilized by the adult male castrates. This was reflected in the cerebral A-V glucose:oxygen ratio of 1.71. Although castration of rats has been shown to release an inhibition at the dehydrogenase level,[7]

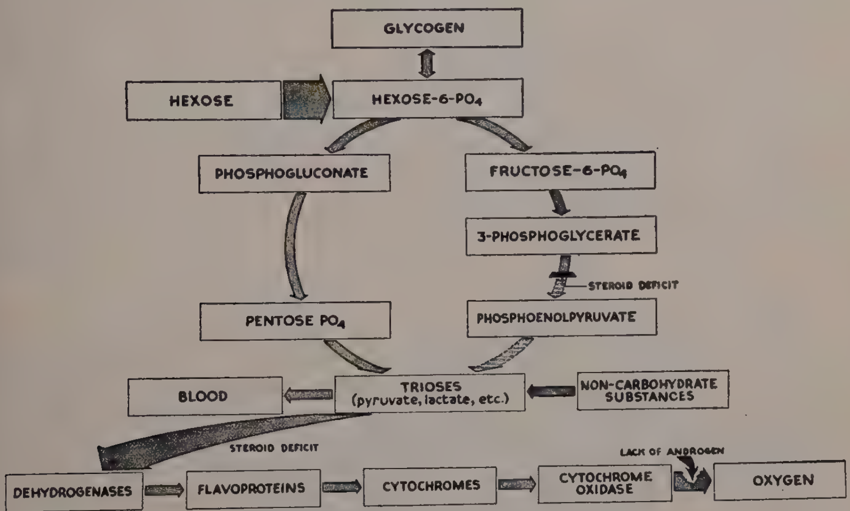

FIGURE 14. Brain of preadolescent eunuchoids.

this might be nullified at another level, as indicated in FIGURE 13. Davis, Meyer, and McShan[106] have presented evidence that the gonadal steroids stimulate cytochrome oxidase activity. These observations could account for the lower rate of cerebral oxygen consumption in the patients with hypopituitarism and the adult male castrates than in the normals. The adult male castrates had received stilbestrol therapy until two weeks before the study. Since McShan and Meyer[4] have shown that cytochrome oxidase activity is inhibited by compounds with terminal phenolic groups, it might be postulated that the stilbestrol therapy resulted in "atrophy" of the cytochrome oxidase of brain. This might account for the fact that the adult male castrates showed the greatest depression of cerebral oxygen consumption among the groups studied.

The reduced oxygen consumption would require an accumulation of trioses. As indicated in FIGURE 13, however, oxidative decarboxylation of glucose-6-phosphate by way of the "glucose-6-phosphate shunt," an aug-

mented deviation of the triose compounds into other metabolic usage, or an increased release of pyruvate or lactate into the cerebral venous blood would abolish this excess.

In contrast, the data indicated a tendency for patients with preadolescent testicular eunuchoidism to have greater than normal rates of *both* glucose and oxygen consumption by the brain. In these respects, their cerebral metabolism was similar to that of the castrate immature rat.[14] FIGURE 14 depicts a scheme that might explain our experimental observations for this group. If, according to Kun's report,[95] a deficiency of gonadal androgens results in some inhibition of the formation of phosphoenolpyruvate, the "glucose-6-phosphate shunt" might function to some degree, as indicated in the figure. Since the cerebral glucose:oxygen ratio of 0.99 for this group does not differ from that obtained for normals (0.97), substrate exchanges within the triose cycle are probably not altered. This suggests that the additional oxygen consumption is used in the oxidation of the glucose increment which could take place in the "glucose-6-phosphate shunt" or in the oxidation of pyruvate. Gonadal steroids, however, have been observed to inhibit the dehydrogenases[7] and also to activate cytochrome oxidase.[106] Disturbance of this balance in favor of release of the steroidal inhibition of the dehydrogenases would be in keeping with the slightly increased oxygen consumption of the testicular eunuchoids.

III. SUMMARY AND CONCLUSIONS

Studies on the actions of steroids upon the brain have been given impetus by the known narcotic action of these compounds, as well as by numerous clinical observations. The ability of steroids to inhibit cerebral respiration *in vitro* assumes physiologic importance in view of the inverse observation that castration of the immature male rat leads to a rapid rate of cerebral metabolism. The increased rate of respiration appears to be specific for brain and can be restored to normal levels by various unsaturated steroids *in vivo* or *in vitro*.

Parallel studies in man *in vivo* demonstrate that clinical endocrine deficiencies are associated with aberrations of cerebral metabolism. Hypopituitarism and adult eunuchism are characterized by a sluggish rate of cerebral metabolism (oxygen consumption). Paradoxically, the brain, in hypopituitarism or eunuchism, utilizes glucose more rapidly than normal, despite the decreased rate of oxygen consumption. Preadolescent eunuchoidism, on the other hand, is characterized by a tendency to increased cerebral utilization of oxygen and glucose. This observation, based on data obtained *in vivo*, is reminiscent of the state demonstrated in the castrate immature male rat by *in vitro* techniques.

The cerebral arterio-venous difference for sugar cannot, unfortunately, be used as an index of the rate of cerebral utilization of sugar after the administration of DCG, since this agent effects a liberation of glucose and galactose from human brain in some instances.

The data demonstrate hormonal, and specifically steroidal, control of cerebral metabolism in man and the rat. Speculative diagrammatic schemes

of possible sites for such controls are submitted as working hypotheses for future investigations.

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STUDIES ON THE INHIBITION OF VARIOUS ENZYMES BY STEROIDS

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Initial investigations on the general problem of the influence of steroid hormones on tissue respiration were undertaken in our laboratories in 1946. The oxidation of glucose by rat liver, kidney, and brain slices was observed in the presence of a variety of steroids. The results obtained in this study were published in 1950.[1] A summary of the changes noted are listed in TABLE 1. Desoxycorticosterone (DOC), dehydroisoandrosterone, methyltestosterone, and testosterone consistently inhibited oxygen consumption at concentrations of 2×10^{-3} M or less. No correlation of the magnitude of these inhibitions with the androgenic, protein anabolic, or renotropic activity of the steroid hormones could be seen. The steroid structure, on the other hand, appeared to determine in a small way the extent of the suppression. Those containing keto groups were, in general, more inhibitory than those with hydroxy groups. Also of interest was the fact that steroids not possessing hormonal activity showed little effect.

Further studies were then carried out with various brain tissue preparations. The oxidation of a number of substrates, including hexoses, phosphorylated intermediates, Krebs cycle components, and amino acids, were tested in the presence of DOC. Results showed an inhibition of at least 30–90 per cent in each instance. This was taken to mean that a reaction or reactions common to all oxidations were being affected. An assay of the cytochrome c–cytochrome oxidase reaction eliminated this system as the site of the DOC action. It thus appeared that the cytochrome reductases or the flavin autoxidizable group might be affected by the steroid. These findings are in general agreement with those of Gordan and Elliott[2] and Eisenberg *et al.*[3]

Since the flavin reductases were a difficult system to prepare and assay, particularly in this case involving an insoluble steroid, D-amino acid oxidase was selected as the model for the elucidation of the nature of the DOC inhibition. The results obtained in this phase of the study were published in 1950.[4]

The Inhibition of D-amino Acid Oxidase by DOC. D-amino acid oxidase is a flavoprotein consisting of two parts, the apoenzyme, a protein molecule which imparts specificity to its function, and a coenzyme, the isoalloxazine adenine dinucleotide (FAD), which functions in transferring hydrogen to oxygen. The products of the oxidation of a D-amino acid are its corresponding keto acids, ammonia, and hydrogen peroxide. The source of the oxidase used throughout this study was an acetone powder of pig kidney. These preparations contained adequate amounts of catalase to decompose the peroxide formed. An extract of our preparation took up 180 μl. of oxygen per hour per 0.7 mg. tissue N in the presence of 0.04 M DL-alanine. All incubations were carried out in Warburg flasks according to the method of Krebs.[5] The steroid was added to the main compartment of the flask in

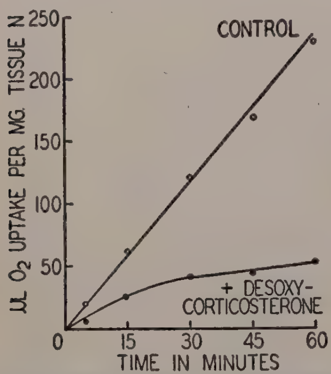
TABLE 1

THE INFLUENCE OF STEROIDS ON OXYGEN UPTAKE OF RAT TISSUE SLICES*

Steroid	Rat liver slices			Rat kidney slices			Rat brain slices		
	Conc. mg./3 ml.	*No. of observ.*	*% Change compared to control*	*Conc. mg./3 ml.*	*No. of observ.*	*% Change compared to control*	*Conc. mg./3 ml.*	*No. of observ.*	*% Change compared to control*
Biologically active									
Testosterone	0.2 –4.0	24	+13 to −47	2.0 –4.0	3	−29 to −42	2.0	2	−33
Testosterone-17-β-diethyl amino ethyl carbonate	1.0 –3.0	4	−11 to −74						
Methyltestosterone	0.2 –2.0	3	−16 to −36	1.0 –2.0	3	−25			
Androsterone	2.2 –4.0	4	−53	3.0	1	−25			
	1.0 –4.0	4	−10 to +15	2.0 –4.0	2	−11 to −14			
Dehydroisoandrosterone	0.2 –3.6	8	−16 to −59	0.2 –2.0	7	−34 to −47	0.5 –2.0	3	−36 to −51
Δ^4-Androstenedione-3,17	1.0 –3.6	4	−10 to −32	2.0	3	−17			
Δ^5-Androstenediol-3(β),17(β)	1.0 –3.2	4	−14 to 0	2.0	2	−15			
Androstanediol-3(α),17(β)	2.0 –3.8	3	−8	2.0	3	−15			
$\Delta^{2 \text{ or } 3}$-Androstenone-3	1.5	1	−4						
Estradiol	0.2 –3.0	7	− 7 to −23	2.0	3	−5			
Estrone	0.2 –3.0	8	+ 4 to −16	0.2 –2.0	4	+ 5 to −33			
Progesterone	0.2 –3.5	5	− 7 to −30	0.2 –3.0	4	−16	2.0	4	−14
Ethynyl testosterone				2.0	2	−4			
Desoxycorticosterone	0.05–3.2	7	−13 to −68	0.05–2.0	6	−16 to −50	0.05–2.0	6	−12 to −60
Biologically inactive									
Cholesterol	2.2 –3.4	3	−3	2.0	2	−9	2.0	2	−2
Pregnanediol-3(α),20(α)	0.2 –1.0	3	+7	2.0	2	−2	2.0	3	−6
Pregnanol-3(α)-one-20	0.2 –3.0	5	−11 to −13	2.0	1	−14			
Allopregnanediol-3(β),20(α)				2.0	3	−17			
Allopregnanediol-3(α),20(α)				2.0	2	−5			

* Glucose 0.001 M, Krebs-Ringer phosphate buffer pH 7.0. Wet weight of tissues per flask: liver, 100–250 mg.; kidney 75 mg.; brain 150 mg. Total volume 3.0 ml. Gas phase—air. 0.2 ml. 20 per cent KOH in center wells. Incubation time, 90 minutes. Steroid additions of less than 1 mg. were pipetted in as the ether solution dried on the bottom of the flasks. Larger amounts were weighed in. (Reprinted from HAYANO, M., S. SCHILLER, & R. I. DORFMAN. 1950. Endocrinology **46:** 387.)

the form of an ether solution and dried in a gentle stream of air. Solutions of DL-alanine and sodium pyrophosphate buffer were then added, and the vessels were chilled before the addition of the enzyme preparation. The flasks and manometers were gassed with oxygen directly after the introduction of the enzyme extract and KOH in the center wells and placed into the 38° bath to be equilibrated for 7 minutes before measuring oxygen uptakes. Under these conditions, complete inhibition of activity is not attained until after some 25 minutes (FIGURE 1). Incubations were usually carried on for an hour, and the percentage inhibition was calculated from the oxygen uptake levels at that time. Incubations continued for as long as 4 hours brought no relieving of the inhibition.



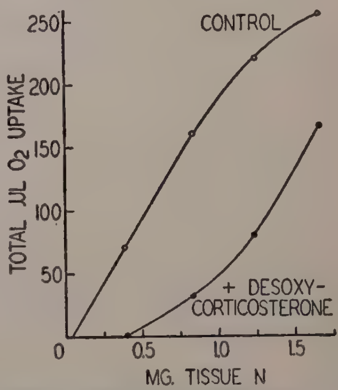


FIGURE 1 (*left*). The effect of incubation time on the inhibition of D-amino acid oxidase by DOC. DL-alanine 0.04 M, sodium pyrophosphate buffer, pH 8.5, 0.017 M, acetone powder extract 0.7 mg. N. DOC 1 mg. Total volume 3.0 ml. (Reprinted from HAYANO, M., R. I. DORFMAN, & E. Y. YAMADA. 1950. J. Biol. Chem. **186**: 603.)

FIGURE 2 (*right*). The effect of enzyme concentration on the inhibition of D-amino acid oxidase by DOC. DL-alanine 0.04 M, sodium pyrophosphate buffer, pH 8.5, 0.017 M. Acetone powder extracts as indicated. DOC 1 mg. Total volume 3.0. Incubation time 60 minutes. (Reprinted from HAYANO, M., R. I. DORFMAN, & E. Y. YAMADA. 1950. J. Biol. Chem. **186**: 603.

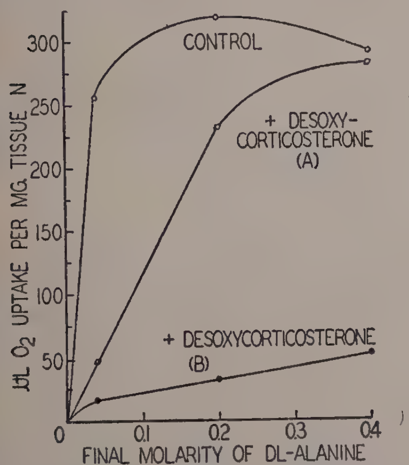
Tissue extract additions were kept between 0.4–0.8 mg. N per flask to insure adequate suppression of the enzyme activity by the level of DOC used. With 0.4 mg. N or less, complete inhibitions are noted even under the conditions of incubation described above. While an increase in enzyme concentration resulted in a decreasing percentage of inhibition, it can be seen that a more or less constant portion of the enzyme activity was inactivated at all levels (FIGURE 2).

That the inhibition was not specific for the substrate DL-alanine was shown by a substitution of DL-methionine and DL-isoleucine. The oxidations of all these amino acids were found to be equally inhibited by DOC.

The mechanism of the inhibition of D-amino acid oxidase activity by DOC was next investigated. To test the possibility that a competition of the substrate and DOC for the oxidase was the cause of the inhibition, the experiment of FIGURE 3 was devised. When excess alanine was present at the start of the incubation, a protection of the enzyme against inhibition was seen, such that, at 10 times the usual concentration of the amino acid,

a complete protection was noted (Curve A). The addition of these large quantities of alanine 15 minutes after the start of incubation, when the inhibition had already been established, brought about essentially no relieving of the inhibition.

A similar experiment was carried out with excess FAD additions to test the possibility of the inhibition resulting from a competition of FAD and DOC for the apoenzyme of the oxidase (FIGURE 4). Here again, additional FAD present at the beginning of the incubation protected the enzyme from inactivation, while additions after the start of incubation brought no relief.



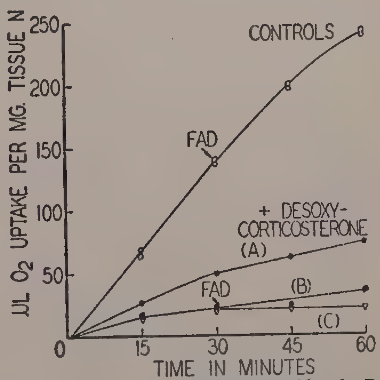


FIGURE 3 (*left*). The effect of alanine concentration on the inhibition of D-amino acid oxidase by DOC. DL-alanine as indicated, sodium pyrophosphate buffer, pH 8.5, 0.017 M. Acetone powder extract 0.64 mg. N. DOC 1 mg. Total volume 3.0 ml. Incubation time 60 minutes. Curve A, alanine concentrations as indicated on the abscissa present in center compartment at zero time. Curve B, 0.04 M DL-alanine present at zero time. Additional alanine to equal final molarities as indicated along the abscissa was tipped in from the side arm 15 minutes after the start of the incubation. O_2 uptake measurements were taken from the time of this addition. (Reprinted from HAYANO, M., R. I. DORFMAN, & E. Y. YAMADA. 1950. J. Biol. Chem. **186:** 603.)

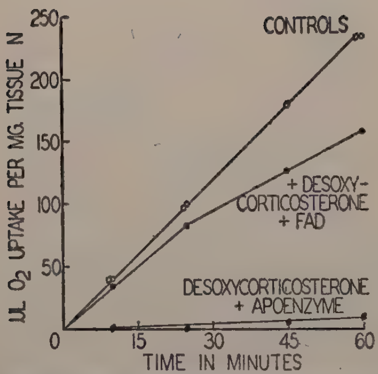
FIGURE 4 (*right*). The effect of FAD on the inhibition of D-amino acid oxidase by DOC. DL-alanine 0.04 M, sodium pyrophosphate buffer, pH 8.5, 0.017 M, acetone powder extract 0.5 mg. N. DOC 1 mg. Total volume 3.0 ml. Curve A, 0.0025, μM FAD was present in the center compartment at zero time. Curve B, 0.015 μM of FAD was tipped in from the side arm after 30 minutes of incubation. Curve C, no additional FAD. (Reprinted from HAYANO, M., R. I. DORFMAN, & E. Y. YAMADA. 1950. J. Biol. Chem. **136:** 603.)

These results thus showed that the inhibition of D-amino acid oxidase by DOC was not one of a competitive type, but one in which the enzyme itself appeared to be affected.

The question of the site of the steroid attack, whether on the protein moiety or on the prosthetic group of the oxidase, was next considered. Parallel incubations of DOC with FAD and DOC with apoenzyme were set up and allowed to react for 20 minutes before completion of the systems, with apoenzyme and alanine in the first instance, and FAD and alanine in the second. The fact that 10 times the usual concentration of alanine protected the enzyme from inactivation but did not relieve an inhibition once established was utilized to advantage here. Under these conditions, only an inhibition occurring in the first 20-minute incubation period should

show up in the final assay. The results obtained are illustrated in FIGURE 5. Essentially no action of DOC on FAD was noted. The DOC-apoenzyme incubation, on the other hand, resulted in a complete inhibition. It was thus concluded that the protein of the oxidase was the point of attack of the steroid.

It was then of interest to determine whether the suppression of activity of the enzyme was the result of an irreversible denaturation of the apoenzyme or whether some enzyme surface phenomena masking or tying up the points of activity was occurring. Two attempts were made to reverse



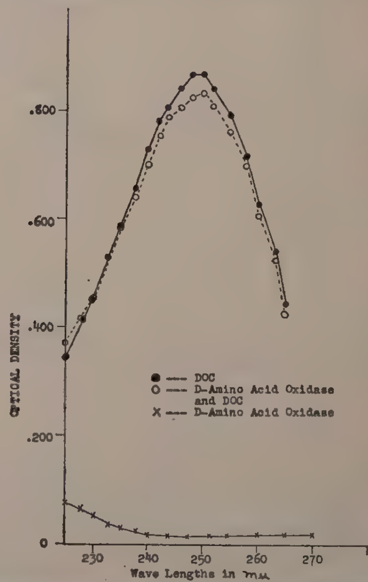


FIGURE 5 (*left*). The effect of DOC on the coenzyme and apoenzyme of D-amino acid oxidase. Final substrate concentrations, DL-alanine, pH 8.5, 0.4 M; pyrophosphate buffer, pH 8.5, 0.017 M; apoenzyme 1 mg.; FAD 0.025 µM. Total volume 3.0 ml. 1 mg. of desoxycorticosterone and FAD or apoenzyme were incubated 20 minutes at 38° before the addition of the other components of the complete system. (Reprinted from HAYANO, M., R. I. DORFMAN, & E. Y. YAMADA. 1950. J. Biol. Chem. **186**: 603.)

FIGURE 6 (*right*). Absorption spectrum of the DOC-inhibited D-amino acid oxidase. DOC 16.7 µG/ml. distilled water. Acetone powder extract 5 µG N/ml. distilled water.

the inhibition. First a dialysis of the inhibited enzyme in a large volume of ice-water was carried out. No relieving of the inhibition was seen (TABLE 2). No attempts were made to dialyze at higher temperatures. A treatment of the inhibited enzyme with acetone in which the preparation was precipitated, filtered, dried, and reassayed resulted in an almost complete regeneration of the oxidase activity. It thus appeared that the inhibitory action of DOC involved the formation of bonds not easily ruptured by aqueous media in which the steroid is poorly soluble but altered by solvents in which the steroid is highly soluble. The DOC-apoenzyme combination appears to be such that no change in the protein structure is evident, in so far as can be concluded by the recovery of the enzyme activity seen here.

No significant change in the structure of DOC is evident in the absorption spectrum of the DOC-inhibited oxidase (FIGURE 6).

The above findings, together with the results seen in a concentration curve of DOC (FIGURE 7), where increasing steroid levels far beyond its aqueous solubility were paralleled by decreasing enzymatic activity, led to the conclusion that an increase in aqueous solubility of DOC by virtue of combination with the apoenzyme must occur. A brief calculation made on the basis of the D-amino acid oxidase purity figures of Negelein and Bromel[6] indicated that the ratio of DOC to the enzyme necessary to bring about an 80–100 per cent inhibition of activity of our preparation was in the neighborhood of 100,000 to 1. It would thus appear that a minute portion of the enzyme portion would serve to solubilize a considerable quantity of steroid. It should be pointed out, however, that the enzyme

TABLE 2

EFFECT OF DIALYSIS AND ACETONE PRECIPITATION ON ACTIVITY OF DOC INHIBITED D-AMINO ACID OXIDASE*

	Non-treated preparation		*Treated preparation*	
	μl. O_2 per mg. N per hr.	*% change*	*μl. O_2 per mg. N per hr.*	*% change*
		Dialysis		
Control	188		144	
Inhibited enzyme	74	−61	54	−63
		Acetone precipitation		
Control	132		133	
Inhibited enzyme	63	−52	125	−6

* Dialysis: Fifteen ml. acetone powder extract incubated with 30 mg. DOC for 30 minutes at 38° and centrifuged to remove any insoluble material. Five ml. was put aside as the non-treated control. Ten ml. was dialyzed against 4 L. of water for 24 hours in the cold. Controls without DOC were run simultaneously.

Acetone precipitation: Enzyme preparations same as above. Ten ml. aliquots were precipitated with 10 volumes of −10° acetone, filtered and resuspended in 5 ml. water for the assay.

Assay: DL-alanine 0.04 M, sodium pyrophosphate buffer, pH 8.5, 0.017 M, 1 ml. above enzyme preparation. Total volume 3.0 ml. Incubation time 60 minutes.

(Reprinted from HAYANO, M., R. I. DORFMAN, & E. Y. YAMADA. 1950. J. Biol. Chem. **186:** 603.)

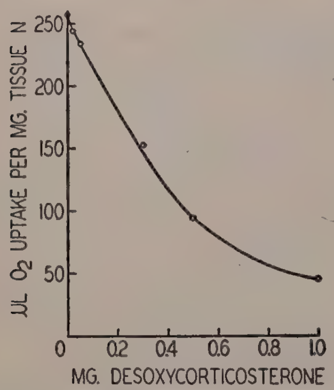
preparation used in these studies was only some 1/120 times as pure as that of Negelein and Bromel, who in turn claimed only a 70 per cent purity for their preparation, and that the presence of other nonspecific protein must be taken into consideration.

It must not be overlooked, however, that insoluble substances such as steroids can combine reversibly with certain specific protein structures and be transported or maintained in a concentrated form in the extra or intracellular fluid where they may exert their action.

The Inhibition of D-Amino Acid Oxidase by Other Steroids. A total of 33 steroids were investigated (TABLE 3). The results showed that DOC was the only steroid in the group of essentially water-insoluble steroids capable of exerting a strong inhibitory effect on D-amino acid oxidase. A more soluble derivative, the glucoside, and compounds similar in structure were less active. Progesterone and 17-hydroxy-11-dehydrocorticosterone were

approximately half as effective. Other insoluble compounds not effective in suppressing the oxidase activity were found to be inhibitory only in a more soluble state as the sodium sulfate ester or after a prolonged incubation with the enzyme in the absence of a substrate. This effect is illustrated in FIGURE 8. A 30-minute exposure of the enzyme to testosterone brought about a maximum of a 50 per cent inhibition.

The Influence of DOC on Other Enzyme Systems. A brief survey of other enzyme systems was made to determine whether DOC inhibited any of these as readily as it did D-amino acid oxidase. Minimum assayable quantities were used with 1 mg. of steroid per 3.0 ml. volume in each case. The results are presented in TABLE 4. Several activities were inhibited



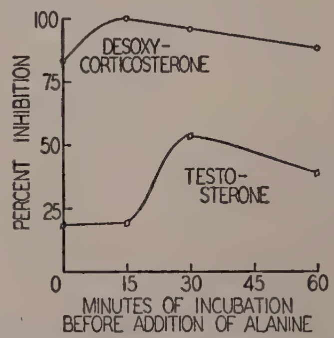


FIGURE 7 (*left*). The effect of DOC concentration on the activity of D-amino acid oxidase. DL-alanine 0.04 M, sodium pyrophosphate buffer, pH 8.5, 0.017 M, acetone powder extract 0.7 mg. N. DOC as indicated. Total volume 3.0 ml. Incubation time 60 minutes. (Reprinted from HAYANO, M., R. I. DORFMAN, & E. Y. YAMADA. 1950. J. Biol. Chem. **186:** 603.)

FIGURE 8 (*right*). The inhibition of D-amino acid by testosterone. DL-alanine 0.04 M, sodium pyrophosphate buffer, pH 8.5, 0.017 M. DOC 1 mg. Testosterone 1 mg. Total volume 3.0 ml. Incubation time 60 minutes. Alanine was added from the side arm at times indicated on abscissa. Oxygen uptake measurements were taken from this time. (Reprinted from HAYANO, M., R. I. DORFMAN, & E. Y. YAMADA. 1950. J. Biol. Chem. **186:** 603.)

by DOC. Among these were tyrosinase, urease, ascorbic acid oxidase, lipase, and transaminase. The inhibition of tyrosinase is of particular interest, in view of the impaired tyrosine metabolism observed in patients with Addison's disease. The activities of two other flavoproteins, L-amino acid oxidase and xanthine oxidase, were not suppressed by DOC. Two enzyme systems were markedly stimulated in the presence of the steroid. In the case of trypsin, the action of DOC appears to be on the substrate, the denatured hemoglobin, since substitution with casein did not result in an increase in activity. With yeast carboxylase, it is our opinion that the action of DOC is on the permeability of the whole yeast cells used in the assay. Further work is anticipated to establish this point.

Summary. The nature of the inhibition of D-amino acid oxidase by desoxycorticosterone (DOC) was studied. The inhibition was found to be due to an action of the steroid on the apoenzyme moiety of the flavoprotein. An increase in the aqueous solubility of DOC by virtue of a combination

with the apoenzyme at a point necessary for enzyme activity appears to occur. Since the inhibition can be relieved by acetone precipitation with a recovery of essentially all the original activity, no change in the apoenzyme

TABLE 3

COMPARATIVE ACTIVITY OF VARIOUS STEROIDS ON D-AMINO ACID OXIDASE

$$\textit{Ratio} = \frac{\textit{Inhibition produced by steroid}}{\textit{Inhibition produced by 1 mg. DOC}}$$

Steroid	*Conc. mg.*	*Mean inhibition % (range)*	*Mean inhibition of DOC (1 mg.) run simultaneously % (range)*	*Ratio = steroid/DOC*
C21 steroids				
Progesterone	1.0	37 (31–44)	82 (68–97)	0.45
Ethynyltestosterone	1.0	5 (3–6)	86 (80–92)	0.06
Desoxycorticosterone acetate	1.0	18 (11–25)	90 (77–100)	0.20
Desoxycorticosterone glucoside	1.0	34 (22–46)	90 (83–97)	0.38
Corticosterone	1.0	14 (3–16)	69 (64–84)	0.20
11-Dehydrocorticosterone	1.0	16	84	0.19
17-Hydroxy-11-desoxycorticosterone	1.0	0 (−9–8)	65 (57–73)	0.0
17-Hydroxycorticosterone	1.0	14 (13–14)	57	0.25
17-Hydroxy-11-dehydrocorticosterone	1.0	35	87	0.40
Allopregnanetriol-3(α),17(α),21-one-20	1.0	6	84	0.07
Androgens				
Sodium androsterone sulfate	1.0	55 (52–57)	85 (78–92)	0.65
	2.0	81	92	0.88
Sodium dehydroisoandrosterone sulfate	1.0	55 (51–58)	85 (78–92)	0.65
	2.0	86	92	0.94
Sodium dehydroisoandrosterone hemi-succinate	1.0	47 (42–51)	80 (80–80)	0.59
Testosterone	1.0	10 (−2–18)	77 (49–100)	0.13
Androsterone	1.0	10 (2–20)	86 (80–92)	0.12
Dehydroisoandrosterone	1.0	12 (5–18)	75 (64–80)	0.16
Δ4-Androstenedione-3,17	1.0	9 (6–11)	71 (49–92)	0.13
Androstanediol-3(α),17(β)	1.0	0 (−5–4)	86 (80–92)	0.00
Δ5-Androstenediol-3(β),17(β)	1.0	7 (2–12)	92 (92–92)	0.08
Methyltestosterone	1.0	−8 (−16–2)	90 (77–100)	−0.08
17(α)-Hydroxyprogesterone	1.0	3 (2–3)	92 (92–92)	0.03

TABLE 3 (*Continued*)

Steroid	*Conc. mg.*	*Mean inhibition % (range)*	*Mean inhibition of desoxycorticosterone (1 mg.) run simultaneously % (range)*	*Ratio = steroid DC*
Estrogens				
Sodium estrone sulfate	0.1	18	80	0.22
	0.2	28	80	0.35
	0.5	58	80	0.72
	1.0	96 (96–96)	80 (80–80)	1.20
	2.0	98	80	1.23
Sodium estradiol sulfate	1.0	86 (76–95)	86 (80–92)	1.00
	2.0	97	80	1.21
Sodium equilen sulfate	1.0	86	64	1.34
	2.0	100	64	1.56
Estradiol	1.0	6 (−1–12)	65 (49–80)	0.09
Estrone	1.0	−2 (−8–5)	65 (49–80)	−0.03
Equilen	1.0	4 (3–5)	86 (80–92)	0.05
Miscellaneous				
Cholesterol	1.0	16 (4–27)	75 (49–100)	0.21
Pregnanediol-3(α),20(α)	1.0	−8 (−5–−10)	70 (49–92)	−0.11
21-Chloroprogesterone	1.0	6 (−12–22)	93 (83–100)	0.07
Δ4-Pregnenediol-17,20-one-3	1.0	20	92	0.22
20,21-Epoxy-Δ4-pregnenediol-one-3	1.0	12 (11–13)	79 (77–80)	0.15
Sodium cholesterol sulfate	1.0	5 (2–8)	72 (64–80)	0.04
	2.0	18	64	0.28

structure is concluded. A survey of the action of other steroids disclosed that DOC alone in the group of essentially water-insoluble steroids was capable of exerting a strong inhibitory effect on the enzyme. Of the other enzyme systems tested, only a few were as readily inhibited as D-amino acid oxidase. These were tyrosinase, urease, ascorbic acid oxidase, lipase, and transaminase. The activities of glutaminase, trypsin, and yeast carboxylase were stimulated by DOC.

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TABLE 4
THE INFLUENCE OF DOC ON OTHER ENZYME SYSTEMS

Enzyme	*Ref.*	*Source*	*Substrate*	*% change in activity range*
D-amino acid oxidase	5	Aqueous extract of pig kidney acetone powder	DL-alanine	−83 (−49 to −100)
Tyrosinase	7	Aqueous extract of potato peelings	Tyrosine	−98 (−95 to −100)
Urease	8	Commercial	Urea	−39 (−27 to −50)
Ascorbic acid oxidase	9	Aqueous extract of squash	Ascorbic acid	−28 (−23 to −32)
Lipase	10	Commercial	Butyrin	−27 (−25 to −28)
Transaminase	11	Saline extract of rabbit heart muscle	Glutamic and oxalacetic acids	−21 (−14 to −28)
Ribonuclease	12	Crystalline enzyme	Yeast nucleic acid	−12 (−6 to −17)
Succinic dehydrogenase	13	Rat liver homogenate	Succinic acid	−10 (−7 to −12)
Arginase	14 8	Purified preparation from beef liver	Arginine	−9 (−6 to −12)
Trypsin	15	Commercial	Casein	−9 (−3 to −15)
Cytochrome oxidase	16	Brain homogenate residue	Phenylene diamine	−9 (−7 to −11)
Cytochrome oxidase	13	Rat liver homogenate	Ascorbic acid	+6 (+3 to +8)
L-amino acid oxidase	17	Venom from *Agkistrodon mokasen*	L-leucine	−6 (−5 to −6)
ATP-ase	18	Rat liver homogenate	ATP	−4 (−2 to −5)
Protease		Rhozyme-DX (Rohm & Haas)	Gelatin	−4 (−4 to −4)
Jack bean carboxylase	19	Jack bean meal	Pyruvic acid	−4 (−9 to +1)
Amylase		Rhozyme-DX (Rohm & Haas)	Starch	−2 (−4 to 0)
Pepsin	20	Commercial	Hemoglobin	0 (−5 to +5)
Acid phosphatase		Polidase-S (Schwarz)	Cocarboxylase	+2 (+1 to +3)
Xanthine oxidase	21	Liver xanthine oxidase	Xanthine	+12 (+10 to +13)
Glutaminase	22	Rat & rabbit kidney homogenates	Glutamine	+26 (+12 to +36)
Trypsin	20	Commercial	Denatured hemoglobin	+73 (+44 to +124)
Yeast carboxylase	23	Bakers' yeast whole cells	Pyruvic acid	+110 (+106 to +116)

Pharmaceutical Products, Inc., Merck & Co., Inc., Endo Products, Inc., and Sharp and Dohme, Inc.

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THE INFLUENCE OF HORMONES ON THE AMINO ACID DEHYDROGENASE SYSTEMS OF THE LIVER AND KIDNEY

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Oxidative deamination is one step in the metabolism of amino acids. This reaction takes place predominantly in the liver and kidney and is under the control of D- and L-amino acid dehydrogenase. Both enzymes are flavoproteins and are group specific: L-amino acid dehydrogenase deaminates L-amino acids, whereas D-amino acid dehydrogenase exhibits strict specificity in oxidizing the "unnatural" D-amino acids. These enzymes catalyze the removal of two hydrogen atoms from the amino acids, resulting in the corresponding imine compounds which, in the presence of water, spontaneously form the corresponding keto acids.

It has become evident that hormones do not initiate any new metabolic processes but rather influence the rate of speed of existing processes. Hormones do not participate in the actual enzymatic processes, *i.e.*, are not essential components of a given enzyme system. They may accelerate or inhibit enzymatic reactions or may cause an increased concentration of a given enzyme in the tissue upon which they act.

This presentation is concerned with a discussion of the available data with regard to the *in vivo* and *in vitro* influence of various hormones on the rate of activity of the amino acid dehydrogenase systems of the liver and kidney.

Determination of Amino Acid Dehydrogenase Activity. Liver or kidney tissue of white male rats of the Sprague-Dawley strain was homogenized with a phosphate buffer of pH 7.35. The oxygen uptake of the extracted homogenate was determined in the presence and absence of DL-alanine, and the difference in the oxygen uptake was taken as the value of amino acid dehydrogenase activity.

Liver Amino Acid Dehydrogenase System

Hypophysis. According to Gaebler, Mathies, and Palm,[1] hypophysectomy in the rat results in a reduced liver D-amino acid dehydrogenase activity. They assumed that the lowering in enzymatic activity was, probably, due to reduced thyroid activity. We did not find any change, however, in the amino acid dehydrogenase activity in the liver of hypophysectomized rats compared to normal (TABLE 1). This difference in results may, perhaps, be due to the difference in testing techniques employed. Gaebler and his associates determined tissue respiration in the presence of DL-alanine, while we took as the value of amino acid dehydrogenase activity the difference in oxygen uptake of tissue homogenate with buffer alone or buffer plus DL-alanine. It is known that hypophysectomy reduces tissue respiration, which can be restored to normal on injection of thyrotrophin or thyroxine.

Since it was found that removal of the adrenal or thyroid gland produces a reduction in the enzyme activity (see TABLE 1), one would assume that

removal of the hypophysis would also result in reduced enzyme activity. That no change in activity was observed may, perhaps, be due to the absence of the pituitary growth principle, which is in agreement with the concept that the growth hormone may inhibit amino acid catabolism either directly or indirectly. It has been shown by Szego and White[2] that growth hormone produces increased fatty acid metabolism and fat deposition in the liver when administered to normal fasted mice. These investigators suggested that the growth hormone may either inhibit amino acid catabolism or accelerate fat metabolism. Russell and Cappiello[3] reported that the rate of urea formation was reduced after the administration of the somatotrophic principle.

TABLE 1
INFLUENCE OF HORMONES ON LIVER AMINO ACID DEHYDROGENASE*

Experimental conditions	*Normal*	*Hypophysectomized*	*Adrenalectomized*	*Thyroidectomized*
	%	%	%	%
Control	100	100	50	50
Epinephrine				
in vivo	60	100	—	—
in vitro	63	—	—	—
ACE				
in vivo	205	—	150	—
in vitro	254	—	—	—
Thyroxine				
in vivo	—	—	—	—
in vitro	100	100	100	—
Insulin				
in vivo	75	—	—	—
in vitro	54	—	—	—
AP Growth Hormone				
in vivo	—	100	—	—
in vitro	—	—	—	—

* Values expressed as % of control value of each group.

The influence of this pituitary principle on the enzyme may be either direct or indirect by promoting increased protein formation from available amino acids and thereby reducing the amount of amino acids available for catabolism. It is generally assumed that the action of the growth hormone is not so much the suppression of amino acid catabolism as the promotion of protein synthesis from available amino acids.

In agreement with Gaebler, Mathies, and Palm,[1] it was observed that treatment of hypophysectomized rats with the anterior pituitary growth hormone did not influence the liver D-amino acid dehydrogenase activity. Apparently, there is synthesis of this enzyme in direct proportion to tissue growth, produced by the growth hormone administration. Gaebler and his associates[1] also found that growth hormone preparations do not influence the liver D-amino acid dehydrogenase of normal rats *in vivo* and *in vitro*.

Adrenal Medulla-Insulin. Epinephrine administration to normal rats was found to cause an inhibition in amino acid dehydrogenase activity,

while in hypophysectomized animals no change in activity was observed (TABLE 1). It is conceivable that the inhibitory effect, observed in normal animals, may be due to the action of insulin, released by epinephrine, on the amino acid dehydrogenase.

Bach and Holmes[4] reported that addition of insulin to liver slices of normal fasted rats inhibits the transformation of certain amino acids into carbohydrate and that this inhibition is accompanied by a reduction in urea formation. Stadie, Lukens, and Zapp[5] found that insulin partially inhibits the deamination by isolated liver slices of normal rats of the unnatural D-isomers of amino acids. The corresponding natural or L-isomers are unaffected. Furthermore, in the complete absence of insulin (removal of the pancreas), deamination by liver slices was found to be much greater than normal. Administration of insulin depressed amino acid dehydrogenase activity. In the hypophysectomized-depancreatized animal (cat), the hypernormal deamination by liver slices found in the depancreatized animal was restored to normal.

The assumption that insulin exerts an inhibitory effect on the liver amino acid dehydrogenase activity is substantiated by our *in vivo* and *in vitro* results, obtained with normal rats (TABLE 1). Colenbrander[6] did not observe any inhibitory *in vitro* effect of insulin on the production of urea and ammonia by liver slices of rats. Enzyme activity was determined, however, without addition of amino acid to the substrate.

Apparently, addition of epinephrine *in vitro* also causes an inhibition (TABLE 1). This effect, however, may not be direct, since it has been found that oxidation of epinephrine by the cytochrome oxidase system results in the production of substances capable of inhibiting oxidase activity.[7]

Adrenal Cortex. TABLE 1 illustrates that normal as well as adrenalectomized rats, given adrenal cortical extract, show an increased oxidase activity of the liver, whereas untreated adrenalectomized animals show a decreased amino acid dehydrogenase activity. An accelerating effect of the secretion of the adrenal cortex was also observed *in vitro* (tissue from normal animals). These findings are in agreement with the assumption that certain factors secreted by the adrenal cortex enhance amino acid catabolism.

It has been suggested[8] that the chief action of the adrenal cortical hormones upon nitrogen metabolism is probably to promote the breakdown of protein to amino acids, rather than to alter the rate of deamination and the formation of urea. Whether the *in vitro* accelerating effect of adrenal cortical extract is specific has still to be investigated.

Thyroid. Klein[9] reported a decrease in the activity of D-amino acid dehydrogenase in the liver of thyroidectomized rats, and an increase in activity when the animals were maintained on an adequate diet which was supplemented with a thyroid preparation. The changes in activity were not associated with a variation in the concentration of the flavin component of the enzyme. We also observed a decreased enzyme activity in the liver of thyroidectomized rats (see TABLE 1). *In vitro* addition of thyroxine to the liver homogenate of normal and hypophysectomized animals did not produce any change in enzyme activity (TABLE 1), while with liver ho-

mogenates from adrenalectomized animals an increase in activity was observed after addition of thyroxine. According to Deane and Greep,[10] thyroidectomy leads to an atrophy of the adrenal cortex. The decrease in enzyme activity in the liver after removal of the thyroid may, therefore, possibly be due to the lowered activity of the adrenal cortex.

Kidney Amino Acid Dehydrogenase System

The amino acid dehydrogenase activity of the kidney seems to be much greater than that of the liver. The amino acid dehydrogenases of the kidney, by the production of ammonia from certain amino acids, may play a role in regulation of acid-base balance. According to Handler, Bernheim, and Bernheim,[11] however, the progressive increase in ammonia ex-

TABLE 2

INFLUENCE OF HORMONES ON KIDNEY AMINO ACID DEHYDROGENASE*

Experimental conditions	*Normal*	*Hypophysectomized*	*Adrenalectomized*	*Thyroidectomized*
	%	%	%	%
Controls	100	70	55	130
Epinephrine				
in vivo	100	142	—	—
in vitro	88	—	—	—
ACE				
in vivo	210	133	—	—
in vitro	—	—	—	—
Thyroxine				
in vivo	—	—	—	—
in vitro	91	100	88	—
Insulin				
AP Growth Hormone				
in vivo	—	115	—	—
in vitro	175	—	—	—

* Values expressed as % of control value of each group.

cretion during experimental and clinical acidosis is not the result of an adaptive increase in the concentration of renal amino acid dehydrogenases.

Hypophysis. Removal of the hypophysis results in a reduction of the kidney amino acid dehydrogenase activity (TABLE 2). Whether this change in activity is due to the atrophy of the adrenal cannot as yet be stated. Administration of growth hormone to hypophysectomized animals results apparently in restoration of enzyme activity from subnormal to normal (TABLE 2). Addition of growth hormone to kidney homogenate from normal rats apparently causes an accelerating effect (TABLE 2). This effect may be nonspecific, however, since it has been found by Wiss and Klingler[12] that D-amino acid dehydrogenase prepared from hog kidney could be activated by the addition of blood proteins.

Adrenal Medulla. Epinephrine administration to normal rats apparently does not influence the enzyme activity, while in hypophysectomized animals an increase in activity was observed (TABLE 2). *In vitro*, epinephrine seems to produce a slight inhibition (TABLE 2). As in the liver homogenate, however, the effect may be indirect.

Adrenal Cortex. Russell and Wilhelmi[13] reported that deamination of DL-alanine by kidney slices from adrenalectomized rats is less than normal. The deamination activity of kidney tissue from adrenalectomized rats may be restored to normal levels or better by the administration to the adrenalectomized animals of adrenal cortical extract or desoxycorticosterone.

These findings are in agreement with our observations of lower kidney amino acid dehydrogenase activity in hypophysectomized and adrenalectomized rats and of the accelerating effect of adrenal cortical extract administration on kidney amino acid dehydrogenase activity in normal and hypophysectomized animals (see TABLE 2).

Thyroid. According to Klein,[9] removal of the thyroid does not influence the activity of the kidney D-amino acid dehydrogenase. However, we observed an increased enzyme activity in kidney homogenates from thyroidectomized rats (see TABLE 2). Apparently, thyroidectomy produces an elevated "resultant" catabolism of amino acids. Such a postulate is in agreement with our observation of a higher concentration of the blood urea in thyroidectomized control rats compared with normal control animals and of the ability of hypophysectomized animals to deaminate injected amino acids at a rate comparable to that observed in normal animals,[14] and with the findings of Persike[15] and of Rupp, Paschkis, and Cantarow[16] that thyroidectomy in the rat is followed by an increased output of urinary N.

Insulin. Stadie, Lukens, and Zapp[5] found that insulin inhibits the deamination of D-amino acids, but not of L-amino acids, in kidney slices. The finding that administration of glucose prevents the fall in blood amino acids associated with a hypoglycemic dose of insulin[17] indicates that loss of nitrogen (by deamination) may be an indirect consequence of diminished utilization of glucose.

Lotspeich[18] has presented evidence to support the conclusion that insulin acts to accelerate the rate at which proteins are synthesized from free amino acids (the influence of the AP growth hormone has to be considered in this connection).

Gonads. Clark, Kochakian, and Fox[19] found that the mouse kidney loses part of its ability to deaminate D-alanine by oxidation as a result of castration. The administration of testosterone propionate not only restored activity but increased it above normal.

Summary

The results thus far obtained seem to indicate that the secretions of the adrenal cortex and the thyroid enhance the amino acid dehydrogenase activity of the liver, whereas insulin inhibits the enzyme activity. With regard to the kidney, the influence of the various hormones has to be studied more extensively before one can draw any definite conclusions.

Influence of the Blood Amino Acid Level on Liver and Kidney Amino Acid Dehydrogenase

Intraperitoneal administration of a casein hydrolysate (Amigen without glucose) to normal, hypophysectomized, and adrenalectomized rats did not

produce any change in the liver amino acid dehydrogenase activity. The effect in thyroidectomized animals was irregular. On the other hand, the kidney amino acid dehydrogenase activity was greatly enhanced under these conditions in normal, hypophysectomized, and adrenalectomized animals, while in thyroidectomized animals a decrease was found (see TABLE 3).

There appears to be a distinction between the factors influencing amino acid dehydrogenase activity of the liver and of the kidney. The amino acid dehydrogenase activity in the kidney apparently is not only dependent upon endocrine factors but may also be directly influenced by the amino acid level of the blood.

In this connection, reference may be made to the finding that maintenance of rats on a high protein diet did not affect the kidney D-amino acid dehydrogenase activity but significantly increased the liver D-amino acid dehydrogenase activity,[20] while a protein-free diet caused a diminution in the liver D-amino acid dehydrogenase activity.[21]

TABLE 3

EFFECT OF CASEIN HYDROLYSATE ADMINISTRATION ON AMINO ACID DEHYDROGENASE*

Tissue	*Normal*	*Hypophysectomized*	*Adrenalectomized*	*Thyroidectomized*
	%	%	%	%
Liver	100	100	100	?
Kidney	148	129	125	80

* Values expressed as % of control value of each group.

General Conclusions

It has been the aim of this review to discuss the relationship of the various hormones to the amino acid dehydrogenase activity of the liver and kidney. It is apparent that the picture which one can draw from the available data is still very incomplete. Our present knowledge of the mechanism by which the endocrine principles may affect enzymatic reactions is rather meager. It should be pointed out that results obtained by *in vitro* experiments may be artifacts and may be without significance in the living animal. Preliminary experiments indicate that there is a relationship between amino acid dehydrogenase activity and phosphate ion concentration. This observation is in agreement with the studies of Kearny and Singer,[22] who found an inhibition of L-amino acid dehydrogenase of snake venom by low concentration of inorganic phosphate.

Caution must also be exercised against making generalizations from results obtained with only one species of animal. For instance, Clark, Kochakian, and Fox[19] have found that, while rat liver brei will form pyruvic acid from D-alanine, mouse liver brei will not and may, therefore, contain no D-amino acid dehydrogenase.

The importance of future investigation in this field is obvious. Results obtained should help in securing a better understanding of the role which hormones may play in the deamination mechanism of amino acids.

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EFFECTS OF STEROIDS AND DIETHYLSTILBESTROL ON DEHYDROGENASE SYSTEMS

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It is the purpose of this communication to describe results which indicate that steroids and diethylstilbestrol possess affinities for dehydrogenase systems that may lead to appreciable effects on enzyme behavior.

Gordan and Elliott[1] originally suggested that the inhibitory action of some steroids and of diethylstilbestrol on oxidations in rat brain tissue homogenates was concerned with the activity of the dehydrogenases present (see: Meyer and McShan[2]).

Hochster and Quastel[3] then showed that diethylstilbestrol was capable of acting as a hydrogen carrier with dehydrogenase systems using manganese dioxide (MnO_2) as the terminal hydrogen acceptor. Under these conditions, diethylstilbestrol was present in the form of its quinone and this quinol-quinone system was considered to possess a carrier function. In the same paper, evidence was presented to show that diethylstilbestrol may compete with cytochrome c as a carrier and that this competition was dependent upon the particular dehydrogenase system under consideration. In the present paper, further evidence is presented to support the theory of carrier competition, the experiments having been carried out using methylene blue and ferricyanide as different hydrogen carriers.

It will be shown, in particular, that the α-glycerophosphate dehydrogenase system of yeast is especially susceptible to the inhibitory action of ketosteroids.

The Manganese Dioxide Technique. Mann and Quastel[4] first used MnO_2 as a hydrogen acceptor in studies of manganese metabolism in soil. Further development of this method was mentioned in our publication on diethylstilbestrol as a hydrogen carrier,[3] and details of the technique are, at present, in process of publication.

Using the conventional manometric apparatus of Warburg, the experiments were carried out in an atmosphere of 93 per cent nitrogen and 7 per cent carbon dioxide at 27° or 37°C., using a medium of 0.025 *M* final concentration of sodium bicarbonate. The following reactions take place, RH_2 representing the substance oxidized:

$$RH_2 + MnO_2 = Mn(OH)_2 + R; \quad (1)$$

$$\text{and} \quad Mn(OH)_2 + CO_2 = MnCO_3 + H_2O. \quad (2)$$

Thus, the principle of the technique depends upon the fact that the velocity of CO_2 uptake is a measure of the rate of MnO_2 reduction by RH_2.

Evidence has been obtained to show that MnO_2 may be linked to several other hydrogen carriers in biological systems. Using the ferricyanide-ferrocyanide, methylene blue-leucomethylene blue and the ferricytochrome c-ferrocytochrome c systems, MnO_2 has been found to be capable of acting as a terminal hydrogen acceptor to each. The final results of these in-

vestigations, the details of which are at present in press, are summarized in FIGURE 1.

The advantages of using this new technique may be summed up as follows: (a) it substitutes anaerobically for cytochrome oxidase, the activity of which is often an important variable; (b) it is a relatively insoluble substance, resembling in this respect cytochrome oxidase itself; (c) in its presence, only traces of carriers need be used, so that possible toxic effects of the carriers themselves on enzyme systems may be easily avoided; and (d) MnO_2 keeps the hydrogen carriers continually in the oxidized form, thus resulting in a more efficient carrier system.

In the work to be described, the MnO_2 technique was used anaerobically, while aerobically the conventional method, using KOH papers, was employed.

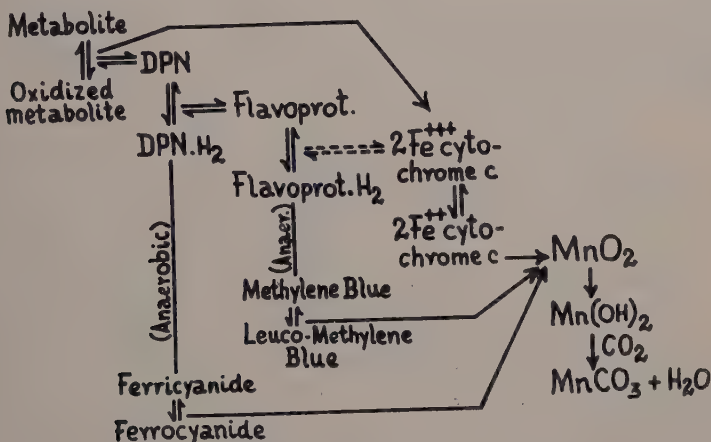

FIGURE 1. Manganese dioxide as a terminal hydrogen acceptor in biological systems.

Competitive Hydrogen Carriers. (*a*) *Anaerobic Studies.* Since the theory of competition between hydrogen carriers was first published,[3] some further observations have been made in this connection. Whereas the early observations were confined to the relationship between cytochrome c and diethylstilbestrol, the present data concern the results obtained when methylene blue or ferricyanide is used as a carrier in place of cytochrome c. The biological material used was a suspension of an acetone-dried powder of yeast (APY), made as described recently.[5] The results are shown in TABLES 1 and 2. It was observed that diethylstilbestrol greatly impedes the carrier action due to methylene blue with all four dehydrogenase systems studied. With ferricyanide as a carrier, however, inhibitions by stilbestrol were observed when the latter was present during the oxidations of alcohol and α-glycerophosphate but not, apparently, of lactate or of triosephosphate.

A summary of the studies with these four enzyme systems and the four carriers used is given in TABLE 3.

(*b*) *Aerobic Studies.* Results given in FIGURE 2 show how the inhibition

Table 1

Effect of Stilbestrol on the Carrier Activity of Methylene Blue in a Yeast-MnO_2 System

System	Substrates (0.0063 *M*)				
	Nil	*Ethyl alcohol*	*Lactate*	*α-Glycerophosphate*	*Hexosediphosphate (NaF)*
APY + Substrate	1	7	4	10	0
APY + Substrate + MeBlue	43	128	268	72	150
APY + Substrate + Stilbestrol	58	104	112	81	127
APY + Substrate + MeBlue + Stilbestrol	53	78	96	53	88

Results in cu. mm. CO_2 uptake in 60 mins.
Concentrations: $NaHCO_3$ 0.025 *M*; MnO_2 suspension 0.2 ml; methanol (as solvent) 1.55 *M*; yeast powder (APY) as suspension 55 mg. per vessel; NaF 0.005 *M*; stilbestrol 0.0023 *M*; methylene blue 0.00008 *M*.
Temperature: 27°C.
Gas phase: 93% N_2 + 7% CO_2.
Final Volume: 3.2 ml.

Table 2

Effect of Stilbestrol on the Carrier Activity of Ferricyanide in a Yeast-MnO_2 System

System	Substrates (0.0063 *M*)				
	Nil	*Ethyl alcohol*	*Lactate*	*α-Glycerophosphate*	*Hexosediphosphate (NaF)*
APY + Substrate	3	7	2	7	0
APY + Substrate + Ferricyanide	43	133	274	92	194
APY + Substrate + Stilbestrol	57	130	103	85	122
APY + Substrate + Ferricyanide + Stilbestrol	48	88	306	78	233

Results in cu. mm. CO_2 uptake in 60 mins.
Concentrations: $NaHCO_3$ 0.025 *M*; MnO_2 suspension 0.2 ml; methanol (as solvent) 1.55 *M*; yeast powder as suspension 55 mg. per vessel; NaF 0.005 *M*; stilbestrol 0.0023 *M*; potassium ferricyanide 0.00187 *M*.
Temperature: 27°C.
Gas phase: 93% N_2 + 7% CO_2.
Final Volume: 3.2 ml.

Table 3

Comparison of the Effects of Carrier Competition by Stilbestrol with Cytochrome c, Methylene Blue, and Ferricyanide in Yeast-MnO_2 Systems

Substrates (all at same final concentration)	Competition by stilbestrol with:		
	Cytochrome c	*Methylene blue*	*Ferricyanide*
Ethyl alcohol	–*	++	++
Lactate	++	++	–
α-Glycerophosphate		++	++
Hexosediphosphate	–	++	–

* – no apparent inhibition; ++ inhibition of more than 50%.

by diethylstilbestrol of aerobic lactate oxidation by a yeast-brain system varies with increasing concentration of the inhibitor.[3] The results are given with both diethylstilbestrol and its quinone, which had been prepared previously by the action of MnO_2 on diethylstilbestrol. These

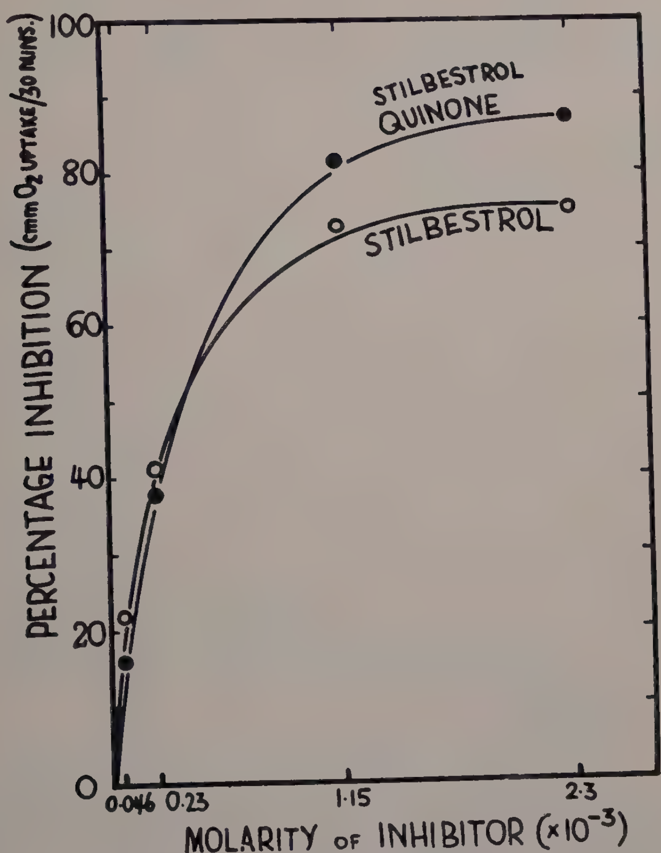

FIGURE 2. The inhibition of the aerobic oxidation of sodium lactate in a yeast-brain system by varying concentrations of diethylstilbestrol and its quinone (cu. mm. O_2 uptake in 30 minutes). Concentrations: phosphate buffer (pH 7.0) 0.022 *M*; yeast powder, 55 mg. per vessel (suspension); fresh rat brain homogenate, 120 mg. wet weight tissue per vessel; nicotinamide, 0.012 *M*; methanol, 1.55 *M* (as solvent for the inhibitors); cytochrome c, 0.000048 *M*; sodium lactate, 0.012 *M*; (quinone prepared by the action of MnO_2 on diethylstilbestrol separately). Temperature: 27°C. Gas phase: air (KOH papers, 0.2 ml. 20% KOH). Total volume: 3.2 ml.

findings give support to the view that diethylstilbestrol and its quinone are capable of forming an equilibrium system.

Effects of Steroids and Diethylstilbestrol on the α-Glycerophosphate Dehydrogenase System. As has been mentioned previously,[3] the acetone-dried yeast powder preparation[3, 5] proved to be a convenient source of yeast dehydrogenase systems. Since the α-glycerophosphate dehydrogenase system of yeast is not influenced by added cozymase, flavoprotein, or cytochrome c and is accelerated poorly by methylene blue,[6] ferricyanide[7] was chosen as a carrier, and this was linked to MnO_2 as described above for the anaerobic experiments.

The addition of ketosteroids to the suspension of the acetone-dried yeast powder oxidizing ethanol, lactate, or triosephosphate had no inhibitory effects. α-Glycerophosphate oxidation, however, was inhibited by some steroids and not by others. This observation led us to a study of a series of steroids, the results of which are shown in FIGURE 3 and TABLE 4. They demonstrate that 3-ketosteroids exercise the highest inhibitory effects whether they contain the αβ-unsaturation in C_3 or not. Some inhibitions were observed with 17- and 20-ketones, whereas the non-ketonic steroids

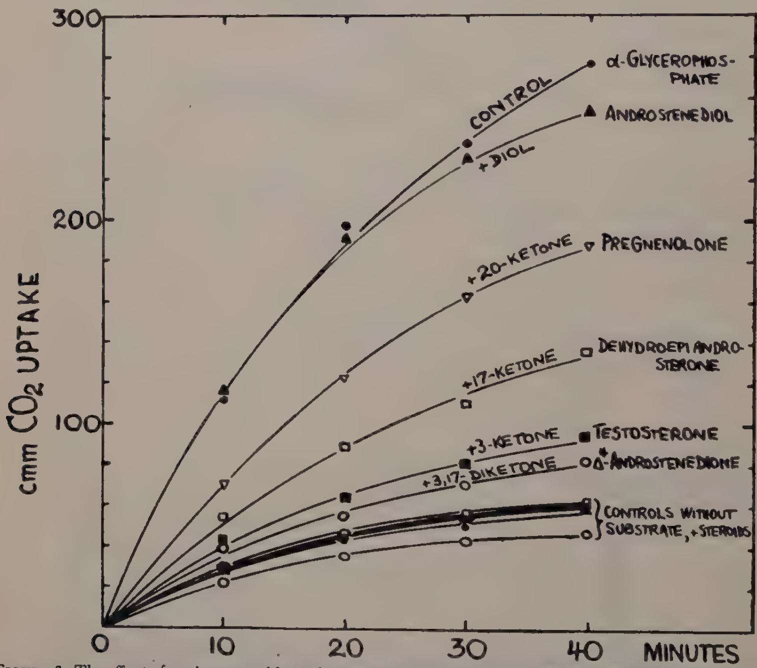

FIGURE 3. The effect of various steroids on the anaerobic oxidation of α-glycerophosphate by yeast catalyzed by ferricyanide (MnO_2 used as a terminal hydrogen acceptor). Concentrations: $NaHCO_3$, 0.025 M; MnO_2, 25 mg. (as suspension); yeast powder, 55 mg. (as suspension); methanol, 1.55 M (as solvent for steroids); sodium α-glycerophosphate, 0.037 M; all steroids, 2 mg.; potassium ferricyanide, 0.0019 M. Temperature: 37°C. Gas phase: 93% nitrogen + 7% carbon dioxide. Total volume: 3.2 ml.

(*e.g.*, Δ^5-androstene-3(β)-17(β)-diol) produced little or no inhibition. It appears that the presence of a C_5 or C_8 side chain significantly alters these effects, usually by causing a diminution of the inhibitory action.

It is difficult to assess the role played by the relative solubility of these molecules in the aqueous medium used, and reference may be made to the demonstration by Hayano, Dorfman, and Yamada[8] that some component of the enzyme system used appeared to increase the solubility of the steroid which they employed. In the case of the bile acids used in our experiments, complete solubility was achieved in the bicarbonate medium and inhibitory effects were observed which were, however, much lower in magnitude than those produced by seemingly much less soluble substances (*e.g.*, Δ^4-androstene-3, 17-dione).

As shown in TABLE 5, the degree of inhibition observed with Δ^4-androstene-3, 17-dione was a function of its concentration and reached a limiting value. (This is analogous to the results observed with diethylstilbestrol in

TABLE 4

INHIBITORY EFFECTS OF STEROIDS AND QUINONES ON THE ANAEROBIC OXIDATION OF α-GLYCEROPHOSPHATE BY YEAST, STUDIED WITH FERRICYANIDE-MnO_2

Compound	*% Inhibition at 60 mins.*	
	27°C 0.009 M substr. concentration	*37°C 0.037 M substr. concentration*
Δ^4-Androstene-3,17-dione	84	82
Androstane-3,17-dione	79	73
Testosterone	86	79
Dehydroepiandrosterone	46	61
Δ^5-Androstene-3(β),17(β)-diol	0	14
Androstane-3(β),17(β)-diol	9	12
Progesterone	80	79
Δ^5-Pregnene-3(β)-ol-20-one	20	42
Desoxycorticosterone acetate	45	38
Estrone	0	21
Estradiol-17(β)	13	38
Cholic acid	35	56
Dehydrocholic acid	17	26
Δ^5-3-Oxy-cholenic acid	54	55
Cholesterol	0	0
Δ^4-Cholestene-3-one	5	0
7-Keto cholesterol acetate	0	2
Diethyl stilbestrol quinone	98	89
2-Methyl-1,4-naphthoquinone	44	71

Concentrations: as in FIGURE 3.

TABLE 5

INHIBITORY EFFECTS OF INCREASING QUANTITIES OF Δ^4-ANDROSTENE-3,17-DIONE AND Δ^5-ANDROSTENE-3(β),17(β)-DIOL ON THE ANAEROBIC OXIDATION OF α-GLYCEROPHOSPHATE BY YEAST

Steroid	*Mg. steroid used and % inhibition*						
	0.1 mg.	*0.5 mg.*	*1.0 mg.*	*2.0 mg.*	*4.0 mg.*	*8.0 mg.*	*16.0 mg.*
Δ^4-Androstene-3,17-dione	18	52	78	82	—	—	—
Δ^5-Androstene-3(β),17(β)-diol	—	—	—	14	13	13	12

the aerobic oxidation of lactate, as shown in FIGURE 2). With androstenediol, however, increases in its concentration did not bring about any increase in inhibition.

The results obtained anaerobically with the ferricyanide-MnO_2 system were confirmed under aerobic conditions when methylene blue was employed

to catalyze the reaction. This is shown in TABLE 6 and it emphasizes the role of the 3-ketone grouping in causing the greatest inhibition of α-glycerophosphate oxidation.

Strong inhibitions of α-glycerophosphate dehydrogenase (TABLES 6 and 7) are also secured by diethylstilbestrol under both anaerobic and aerobic conditions.

The studies of α-glycerophosphate oxidation were then extended to animal

TABLE 6

EFFECTS OF STEROIDS AND DIETHYL STILBESTROL ON THE AEROBIC OXIDATION OF α-GLYCEROPHOSPHATE BY YEAST, CATALYZED BY METHYLENE BLUE

Compound	*Presence or absence of 3-keto group*	*% Inhibition at*	
		60 mins.	*120 mins.*
Δ^4-Androstene-3,17-dione	+	48	58
*Δ^5-Androstene-3,(β),17(β)-diol	−	0	0
Dehydroepiandrosterone	−	0	5
Testosterone	+	52	46
Progesterone	+	51	71
*Δ^5-Pregnene-3(β)-ol-20-one	−	0	0
*Cholesterol	−	0	0
Diethyl stilbestrol		100	90

* In these cases, some acceleration of the system was observed.
Concentrations: phosphate buffer (pH 7) 0.015 *M*; methylene blue. 0.00008 *M*; methanol 1.55 *M* (as solvent for the compounds used); compounds used 2 mg.; sodium α-glycerophosphate 0.037 *M*; yeast powder 60 mg. (as suspension).
Temperature: 37°C.
Gas Phase: air (KOH papers, 0.2 ml. 20% KOH).
Total Volume: 3.3 ml.

TABLE 7

EFFECTS OF STEROIDS AND DIETHYL STILBESTROL ON THE ANAEROBIC OXIDATION OF α-GLYCEROPHOSPHATE BY A RAT LIVER HOMOGENATE IN THE PRESENCE OF DPN, NICOTINAMIDE, FERRICYANIDE, AND MnO_2 AS THE TERMINAL HYDROGEN ACCEPTOR

Compound	*Presence or absence of a*			*% inhibition at 60 mins.*
	3-ket.	*17-ket.*	*20-ket.*	
Δ^4-Androstene-3,17-dione	+	+	−	43
Δ^5-Androstene-3(β),17(β)-diol	−	−	−	9
Dehydroepiandrosterone	−	+	−	22
Testosterone	+	−	−	48
Progesterone	+	−	+	70
Δ^4-Pregnene-3(β)-ol-20-one	−	−	+	25
Cholesterol	−	−	−	0
Diethyl stilbestrol				42

Concentrations: $NaHCO_3$ 0.025 *M*; MnO_2 25 mg. (as suspension); DPN 0.001 *M*; nicotinamide 0.03 *M*; methanol 1.55 *M* (as solvent); compounds used 2 mg.; sodium α-glycerophosphate 0.037 *M*; potassium ferricyanide 0.0019 *M*; liver tissue 575 mg. wet weight (as homogenate in saline-nicotinamide).
Temperature: 37°C.
Gas phase: 93% Nitrogen + 7% carbon dioxide.
Total Volume: 3.2 ml.

tissues. As the results in TABLES 7 and 8 show, a similar phenomenon was observed with rat liver homogenates, but the inhibitions were not nearly so pronounced as in the yeast system. When a brain homogenate was used as the source of the enzyme system, no significant inhibitory effects were observed in the oxidation of α-glycerophosphate with the same ketosteroids.

Also, an extract of a rabbit skeletal muscle powder, made as described by Green, Needham, and Dewan,[9] contained a very active α-glycerophosphate oxidizing enzyme which was not inhibited by the ketosteroids.

The Effect of Rat Tissue Homogenates on the Inhibition by Androstenedione of the Anaerobic Oxidation of α-Glycerophosphate by Yeast. It seemed possible that the lack of inhibitory effects of steroids on α-glycerophosphate oxidation in animal tissues might be due to the presence in these tissues of

TABLE 8

EFFECTS OF STEROIDS AND DIETHYL STILBESTROL ON THE AEROBIC OXIDATION OF α-GLYCEROPHOSPHATE BY A RAT LIVER HOMOGENATE, IN THE PRESENCE OF ADDED DPN, NICOTINAMIDE, AND CYTOCHROME C

Compound	*Presence or absence of a*			*% Inhibition at*	
	3-ket.	*17-ket.*	*20-ket.*	*60 mins.*	*120 mins.*
Δ^4-Androstene-3,17-dione	+	+	−	24	29
*Δ^5-Androstene-3(β),17(β)-diol	−	−	−	0	0
Dehydroepiandrosterone	−	+	−	24	19
Testosterone	+	−	−	36	33
Progesterone	+	−	+	24	35
Δ^4-Pregnene-3(β)-ol-20-one	−	−	−	24	22
*Cholesterol	−	−	−	0	0
Diethyl stilbestrol				100	100

* In these cases, some acceleration of the system was observed.
Concentrations: phosphate buffer (pH 7) 0.015 *M*; nicotinamide 0.03 *M*; DPN 0.001 *M*; cytochrome c 0.000048 *M*; sodium α-glycerophosphate 0.037 *M*; methanol 1.55 *M* (as solvent); compounds used 2 mg.; liver tissue 575 mg. wet weight (as homogenate, prepared in saline-nicotinamide).
Temperature: 37°C.
Gas Phase: air (KOH papers, 0.2 ml. 20% KOH).
Total Volume: 3.3 ml.

TABLE 9

EFFECT OF RAT TISSUE HOMOGENATES ON THE INHIBITION BY ANDROSTENEDIONE OF THE ANAEROBIC OXIDATION OF α-GLYCEROPHOSPHATE BY YEAST (STUDIED WITH THE AID OF THE FERRICYANIDE-MnO_2 SYSTEM)

System: $NaHCO_3$ 0.025 *M; MnO_2* 33 *mg.; MeOH* 1.55 *M; yeast powder* 55 *mg.; ferricyanide* 0.0019 *M* *Plus:*	*cmm. CO_2 uptake in 60 minutes*			
	no additions	*+ brain homog.* (110 mg. tissue wet weight)*	*+ heated brain homog.† (110 mg. tissue wet weight)*	*+ liver homog.* (110 mg. tissue wet weight)*
Nil	93	166	163	195
Androstenedione (2 mg.)	87	125	113	104
α-Glyceroph. (0.037 *M*)	298	442	398	302
α-Glyceroph. + Androstenedione	126	394	324	148
% Inhibition	80.9	2.5	10.2	58.8

* Homogenized in saline.
† Homogenized in saline, heated in a boiling water bath for 30 minutes

components capable of modifying the action of the steroids. If this were the case, the addition of a small amount of an animal tissue homogenate to a yeast system might prevent the ketosteroids from exercising their normal inhibitory effects. This was found to be the case, and the results are shown in TABLE 9. They show that the presence of a brain homogenate dimin-

ishes the steroid inhibition of the yeast system, that heating the brain preparation has but little effect on this diminution, and that liver tissue is much less effective than brain.

When the relative quantities of steroid and brain tissue were varied, it was found that, for increasing quantities of steroid, increasing amounts of

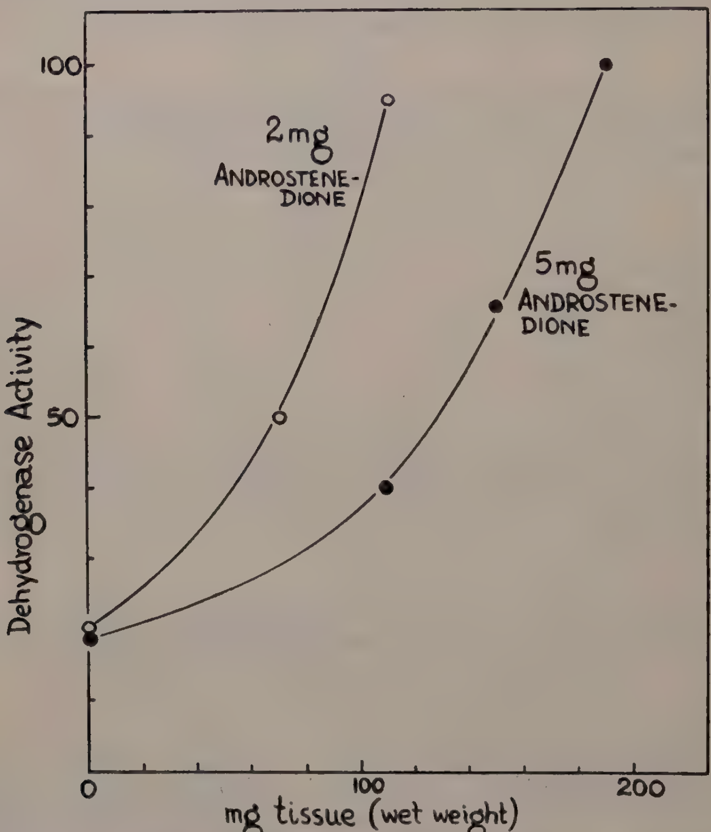

FIGURE 4. Effect of increasing quantities of a rat brain homogenate on the ability of androstenedione to inhibit the anaerobic oxidation of α-glycerophosphate by yeast. Concentrations: as indicated in TABLE 9.

brain tissue were required before a neutralization of the inhibition of the yeast system was obtained. Typical results are shown in FIGURE 4.

Summary. It has been shown that 3-ketosteroids can act as strong inhibitors of the aerobic and anaerobic oxidation of α-glycerophosphate by yeast, while 17- and 20-ketones inhibit to a lesser extent. The presence of a C_{17} side chain appears to prevent the inhibition which would otherwise be caused by a C_3-ketone contained in the same molecule. It has also been shown that a brain homogenate is capable of neutralizing the inhibition of a steroid on this yeast dehydrogenase system. It seems possible that the inability of the ketosteroids to inhibit animal tissue α-glycerophosphate oxidation is the result of the presence of components in these tissues which

may combine preferentially with the steroid before the latter can exercise its effect.

Evidence has been presented also to show that diethylstilbestrol is capable of acting as a competitive hydrogen carrier to the action of methylene blue or ferricyanide in yeast dehydrogenase systems.

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METABOLIC EFFECTS OF THYROXINE *IN VITRO**

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Studies of the mechanism by which the hormone of the thyroid gland exerts its metabolic effects must of necessity be designed to obtain information relevant to several different questions. First of all, it is necessary to learn the chemical nature of the metabolically active form of the hormone, and it is desirable to know which modifications of the structure can be made without loss of activity and which result in complete loss of activity. Secondly, it is necessary to learn the level of protoplasmic organization at which the hormone exerts its influence. The final question is, by what exact mechanism is the influence exerted? Biochemists, currently, want this answer in terms of effects of the hormone on enzymatically catalyzed reactions.

The studies of Taurog and Chaikoff[1] and of Laidlaw[2] lead us to believe that thyroxine itself is the circulating form of the thyroid hormone. Whether thyroxine undergoes metabolic and structural changes within the cells it influences before it becomes the active hormone is still not known. Rather wide deviations from the structure of thyroxine are permissible without complete elimination of hormonal activity.[3] Neimann and coworkers[4] have interpreted the biological activity[5] of their synthetic isomers of thyroxine as indicating that only those structures capable of oxidation to a quinoid form have hormone activity. This renewed interest in the concept long championed by Kendall[6]: that thyroxine participated in oxidation-reduction systems.

In regard to the level of organization required to demonstrate metabolic effects of the hormone, considerably more data are available. An increased rate of oxygen consumption has been observed in a number of different organs removed from animals which had previously been given relatively large large doses of thyroxine or thyroid substance. The response is still apparent in tissue slices and in several cases has been traced to an actual increase in the tissue content of certain enzymes. A considerable time interval is needed, following administration of the hormone, before such responses can be observed. When thyroxine is applied directly to surviving tissue *in vitro*, less consistent results are obtained. To cite an example, Davis, Da Costa, and Hastings[7] found that the addition of thyroxine to excised, intact frog heart increased the rate of respiration, but if the heart was sliced no effect was obtained. Some workers[8, 9] have found extremely low concentrations of thyroxine to accelerate methylene blue reduction by minced muscle. The comparatively few reports of thyroxine accelerating oxygen consumption of minced tissue seem not to have been confirmed.[10]

As far as the mechanism of thyroxine's action on enzyme systems is concerned, everything remains to be discovered. Our experiments in this field began in 1942, when we attempted to confirm the report of Carter[11] that thyroxine increased the respiration of rabbit spermatozoa. We found that

* The work reported in this paper was supported by a grant from the Nutrition Foundation, Inc.

thyroxine inhibited respiration, and slightly stimulated glycolysis, of bovine spermatozoa.[12] Nor could we confirm Carter's results with rabbit spermatozoa. Carter, however, employed a suspension medium which contained considerable amounts of calcium—a substance having a pronounced deleterious effect on respiration, glycolysis, and motility of spermatozoa.[13]

The effect of thyroxine on spermatozoa of various species was therefore tested in the presence and absence of calcium. It was found to have no

TABLE 1

EFFECT OF CALCIUM AND THYROXINE ON RESPIRATION OF RAM SPERMATOZOA

Expt. no.	Sperm conc. per flask	Calcium^{++}	Thyroxine	Z_{O_2}					
				control	plus calcium^{++}	plus thyroxine	calcium and thyroxine	% inhibition by calcium^{++}	% reversal of inhibition by thyroxine
	$\times 10^8$	$\times 10^{-3}$ M	$\times 10^{-5}$ M						
1	1.57	6	2	34.9	20.3		31.8	42	79
2	2.11	6	2	14.6	4.7	14.8	6.7	68	20
3	2.24	4	2	9.6	5.3		7.8	45	58
4	1.73	6	1	14.9	6.4		7.4	57	16
		6	2		6.4	11.5	8.5	57	25
		4	2		2.6		4.0	83	16
5	1.75	4	2	15.3	6.9	11.4	11.8	55	58
		6	1		5.0	11.0	12.1	67	69
		6	2			11.4	13.8		85
		6	3				11.1		59
		6	4			15.3	10.8		57
6	2.05	4	2	14.1	0.0	14.3	8.4	100	60
		6	1		2.8	14.7	7.8	80	44
		6	2			14.3	8.8		53
		6	3				9.5		59
		6	4			22.3	6.3		31
7	2.70	4	1	7.6	5.2		4.7	31	−21
		4	2				2.7		−100
8	3.50	4	1	17.4	15.1		16.5	13	61
		4	2			17.0	14.3		−35
		6	1		10.0		11.3	42	14
		6	2			17.0	14.5		61

Z_{O_2} = cu. mm. O_2/100 millon cells/hr.
Respiration, in absence of added substrates, was measured at 37°.

appreciable effect on the spermatozoa of most species (including a few specimens of rabbit spermatozoa) but seemed beneficial in reversing calcium inhibition of ram spermatozoa. The data for ram spermatozoa, obtained by Dr. D. Ghosh, are shown in TABLE 1. Thyroxine alone inhibited respiration slightly in some of the experiments, but it enhanced respiration in the majority of experiments when calcium was present. It seems likely that it was this effect that Carter[11] observed, for his "control" rate of respiration was, in reality, the rate of calcium-inhibited spermatozoa.

The studies were then extended to homogenates of rat liver and kidney. Thyroxine was not effective in reversing calcium inhibition in these systems

but seemed to have a slight inhibitory action of its own, especially with the kidney preparations. A summary of the effect of thyroxine on the oxidation of glutamic acid and various Krebs cycle intermediates is presented in TABLE 2. Respiration was measured at 37° by conventional manometric technique. The system contained 50 μ moles phosphate buffer pH 7.4, 10-15 μ moles $MgSO_4$, 0.5 μ mole cytochrome c, 6 μ moles ATP, 30 μ moles of oxidizable substrate, 0.2 ml. of a 30 per cent suspension of the twice washed residue of rat kidney homogenate, and isotonic KCl to make 3.0 ml.

Thyroxine,* at 1.3×10^{-6} M, inhibited glutamate oxidation in all experiments. With other substrates, inhibition was obtained less frequently and was usually less pronounced when it did occur. At higher concentrations, thyroxine inhibited oxidation of all these substrates, but its effect was

TABLE 2

INFLUENCE OF THYROXINE ON THE OXIDATION OF VARIOUS KREBS CYCLE INTERMEDIATES AND AMINO ACIDS BY WASHED RESIDUE OF RAT KIDNEY HOMOGENATE

Substrate	$(Q_{O_2}N)$* *Molarity of thyroxine*			*% Inhibition by 1.3 × 10^{-5} M thyroxine*
	none	*1.3 × 10^{-6}*	*6.5 × 10^{-6}*	
Glutamate	502	424	312	57 (29)†
α-Ketoglutarate	544	502	490	28 (7)
Succinate	540	540	502	31 (6)
Fumarate	220	226	168	26 (4)
Pyruvate	500	496	520	14 (6)
Citrate	326	330	312	23 (9)
cis-Aconitate	254	208	202	23 (8)
Proline	346		366	0 (1)

* Q_{O_2} (N) = cu. mm. oxygen consumed per hour per mg. enzyme nitrogen. In the table, the data are calculated from the first 30 minutes of the experimental period—T = 37°. The substrates were added to give a final concentration of 0.01 M. Other additions are described in the text.
† Numbers in parentheses represent the number of experiments.

always greatest on glutamate. The oxidation of various substrates by washed residues of rat liver homogenate was not inhibited by these same concentrations of thyroxine.

Effects of Thyroxine on Glutamate Oxidation by Rat Kidney Enzymes. The structural specificity of thyroxine as an inhibitor of glutamate oxidation has been studied with all available isomers and analogs. Natural *L*-thyroxine, kindly provided by Prof. E. P. Reineke of Michigan State College, was no more effective than *DL*-thyroxine, indicating that the *D* isomer is equally inhibitory (TABLE 3). The inhibitory action of several structural isomers and analogs of thyroxine is shown in TABLE 4. Only those compounds which have some thyroxine-like activity in intact animals[3,5] inhibited glutamate oxidation when tested at 1.3×10^{-5} M. The effectiveness of these isomers and analogs in inhibiting glutamate oxidation, however, was not correlated with their *relative* thyroxine-like activity in intact animals. For example, ortho-thyroxine, which inhibited glutamate oxidation as

* Solutions of thyroxine were prepared as described previously.[12] The effects of thyroxine solutions described in this paper are not obtained with equivalent amounts of dilute NaOH.

strongly as did thyroxine, is only $\frac{1}{25}$th as active in stimulating BMR of the thyroidectomized rat.[5]

The diiodobenzoic acid analog (Compound 5) gave only a fleeting inhibition during the first 10 to 15 minutes of the experiment. Frieden and Winzler found this compound to be only slightly active in stimulating metamorphosis of tadpoles.[3] Thyronine and diiodotyrosine did not inhibit glutamate oxidation at the concentrations tested. Likewise, thyroglobulin[14] was completely inactive in our *in vitro* systems.

These results seemed to indicate that the *in vitro* action of thyroxine was relatively specific and that further studies were justified.

FIGURE 1 describes the time course of the effect of thyroxine on glutamate oxidation. Thyroxine exerted a strong initial inhibition which was much less apparent during the second hour of the experiment. Thyroxine, however, adversely affected maintenance of respiration by the kidney preparation regardless of the substrate added originally. Both the specific inhibition of glutamate oxidation and the deleterious effect of thyroxine on

TABLE 3

COMPARATIVE EFFECT OF *L*- AND *D,L*-THYROXINE ON GLUTAMATE OXIDATION

Concentration	$Q_{O_2}(N)$*	
	DL-thyroxine	*L-thyroxine*
0	420	420
1.3×10^{-6}M	410	420
6.5×10^{-6}M	380	385
1.3×10^{-5}M	254	296
2.6×10^{-5}M	222	226

* Conditions as in TABLE 2.

maintenance of the respiratory system are more apparent at low concentrations of the enzyme preparation. As shown in FIGURE 2, the inhibitory effect of thyroxine diminishes as the enzyme concentration is increased. The lesser inhibitory effect of thyroxine on the oxidation of α-ketoglutarate was more readily overcome by increasing quantities of enzyme.

Since the washed residue preparations represent a relatively uncontrolled mixture of cellular components, it was decided to study the effect of thyroxine on more precisely defined tissue fractions. Centrifugal fractionation of the cellular components of rat kidney homogenized in isotonic (.25M) sucrose was carried out by the procedure of Schneider.[15] As shown in FIGURE 2, the mitochondrial fraction was less susceptible to thyroxine inhibition, and increasing concentrations of the mitochondria completely overcame the inhibition of glutamate oxidation.

The decreased susceptibility of the mitochondrial fraction to the inhibitory effect of thyroxine seems to be related to the relative absence of other cellular constituents and not merely to the fact that sucrose replaced KCl as the homogenizing medium. The oxidation of glutamate by the whole homogenate (in 0.25 M sucrose) or by fractions containing large quantities of nuclei is readily inhibited by thyroxine.[16]

TABLE 4

INHIBITION OF GLUTAMATE OXIDATION BY COMPOUNDS STRUCTURALLY RELATED TO THYROXINE

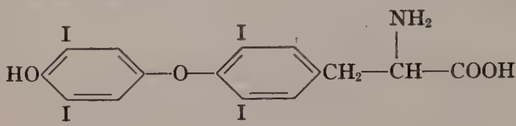
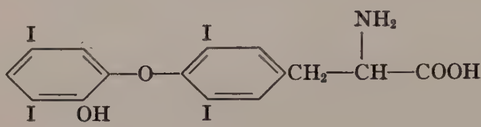
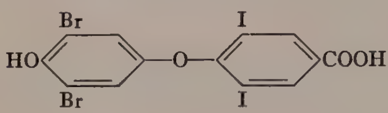
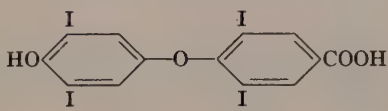
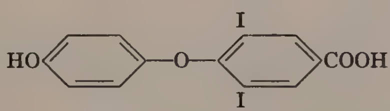
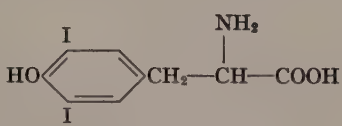
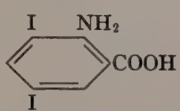
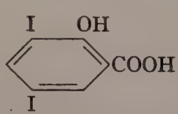
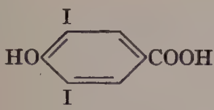
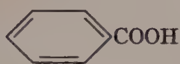
		% Inhibition at 1.3 × 10^{-5} M	Other assays*
1.		37	++++
2.		36†	+
3.		49‡	++
4.		32‡	++
5.		2‡	+
6.		0	–
7.		0	–
8.		0	–
9.		0	–

TABLE 4—*Continued*

		% Inhibition at 1.3 × 10⁻⁵ M	*Other assays**
10.		0	—
11.		4	—
12.		0	—
13.		7	—
14.		0	—

* See references (3) and (5).
† Kindly supplied by Prof. Carl Niemann.
‡ Kindly supplied by Dr. Earl Frieden and Prof. R. J. Winzler.

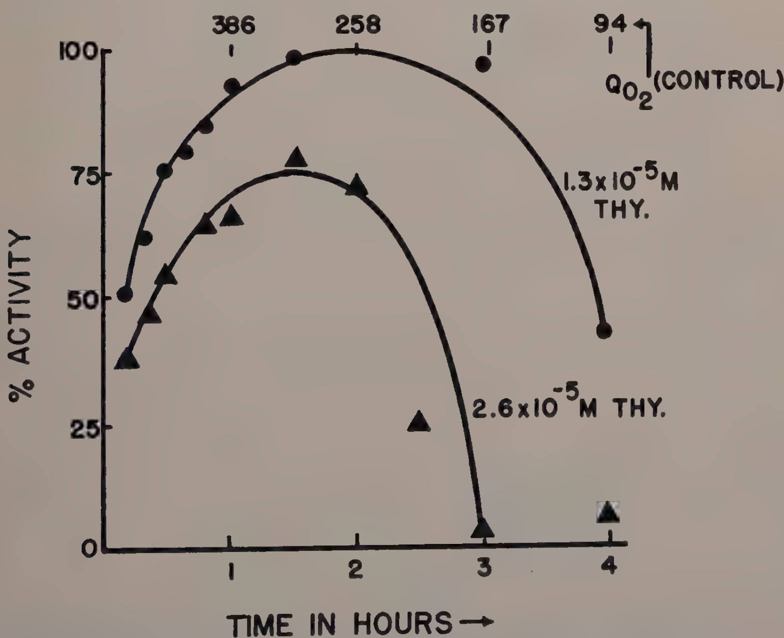


FIGURE 1. The effect of time on thyroxine inhibition of glutamate oxidation. Conditions as in TABLE 2. 0.9 mg. N per flask. The figures across the top show the rate of respiration in the control flasks.

The intact structures of mitochondria are not necessary to demonstrate the effect of thyroxine on glutamate oxidation. The hormone inhibits the one-step oxidation of glutamate to α-ketoglutarate in the glutamic dehydro-

genase assay procedure of Copenhaver *et al.*,[17] where a water homogenate is employed as the enzyme source. As shown in TABLE 5, higher concentrations of thyroxine are required to obtain a given degree of inhibition with this system than in the washed residue of an isotonic KCl homogenate.

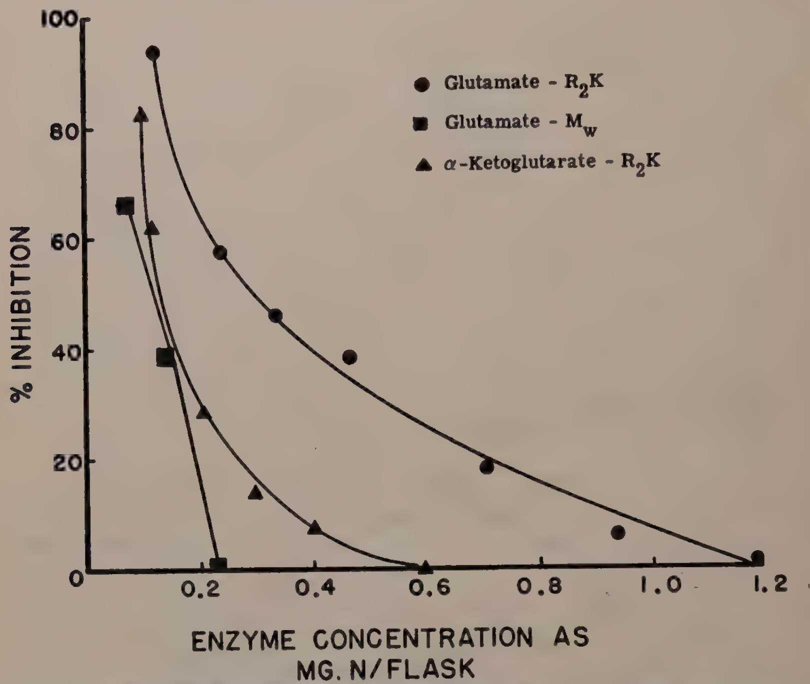


FIGURE 2. Effect of enzyme concentration on thyroxine inhibition. The data show the inhibition by 1.3×10^{-5} M thyroxine during the first hour of the experiment at 37°. R_2K = twice washed residue of rat kidney homogenate (KCl). M_w = twice washed mitochondria from a sucrose homogenate of rat kidney.

TABLE 5
EFFECT OF THYROXINE ON GLUTAMIC DEHYDROGENASE OF RAT KIDNEY

	Q_{O_2} (N)
Control	107
Control plus 1.3×10^{-6}M thyroxine	113
Control plus 4.0×10^{-6}M thyroxine	115
Control plus 6.5×10^{-6}M thyroxine	100
Control plus 1.3×10^{-5}M thyroxine	99
Control plus 2.6×10^{-5}M thyroxine	64

This may result from the protective effect of diphosphopyridine nucleotide (DPN), which is added to the glutamic dehydrogenase assay system at a concentration of 2 mg./ml.

The protective effect of DPN is apparent also with washed residue preparations and glutamate as the substrate (TABLE 6). The oxidation of glutamate by the washed kidney residue is appreciably enhanced in the

presence of DPN (see also Kornberg and Lindberg[18]). The response of the system to thyroxine varied a great deal when DPN was present, but, in general, thyroxine was much less inhibitory when the coenzyme was present.

Decreasing the concentration of ATP or substituting adenylic acid for

TABLE 6

EFFECT OF DPN AND THYROXINE ON THE OXIDATION OF GLUTAMATE

Additions	Q_{O_2} (N)								
	Control			*1.3 × 10⁻⁵ M thyroxine*			*2.6 × 10⁻⁵ M thyroxine*		
	1st hr	*2nd hr*	*3rd hr*	*1st hr*	*2nd hr*	*3rd hr*	*1st hr*	*2nd hr*	*3rd hr*
None	386	258	167	278	254	153	205	192	25
3 mg. DPN	514	338	214	558	377	246			
6 mg. DPN	517	343	224				319	83	41
None	473	345	225	363	322	205			
3 mg. DPN	585	384	215	498	358	216			
6 mg. DPN	500	341	185	468	367	162			

TABLE 7

COMPARATIVE EFFECTS OF ATP AND AMP ON THYROXINE INHIBITION OF GLUTAMATE OXIDATION BY A RAT KIDNEY RESIDUE

Hr.	*c.mm. O_2 Consumed*											
	1×10^{-3} M ATP			*2×10^{-3} M ATP*			*1×10^{-3} M AMP*			*2×10^{-3} M AMP*		
	control	*thyroxine*		*control*	*thyroxine*		*control*	*thyroxine*		*control*	*thyroxine*	
		1.3×10^{-5} M	*2.6×10^{-5} M*		*1.3×10^{-5} M*	*2.6×10^{-5} M*		*1.3×10^{-5} M*	*2.6×10^{-5} M*		*1.3×10^{-5} M*	*2.6×10^{-5} M*
1	281	245	176	353	300	222	274	189	109	368	242	141
2	209	177	55	279	248	198	170	13	4	239	177	25
3	107	30	5	224	187	151	34	5	5	165	28	4
4	13	6	0	171	130	46	3	0	0	50	3	2

ATP = Adenosine triphosphate. AMP = Adenosine monophosphate (muscle adenylic acid).

ATP enhanced the inhibitory effect of thyroxine on glutamate oxidation (TABLE 7).

The relationship of glutamate concentration to the inhibitory effect of thyroxine is shown in FIGURE 3. In the control flasks, respiration was constant at all substrate levels above 10 μ moles per flask. Glutamate competitively reversed the inhibitory effect of thyroxine. When plotted in the conventional Lineweaver-Burk manner, however, the data do not indicate a *simple* competitive relationship. Further studies are being made on this point.

Effect of Thyroxine on Fatty Acid Oxidation. Since our first studies of the effects of thyroxine were made on the lipid oxidizing[19] system of spermatozoa,

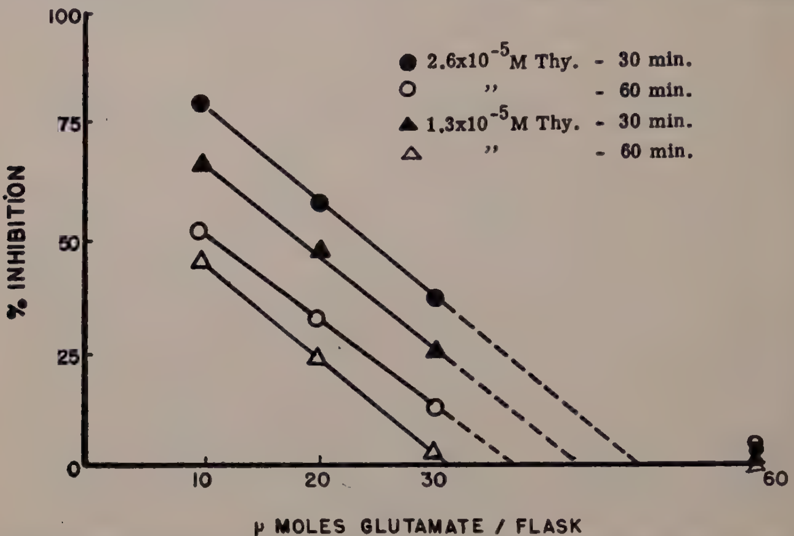


FIGURE 3. Effect of glutamate concentration on thyroxine inhibition (0.86 mg. of N as washed residue o rat kidney homogenate per flask).

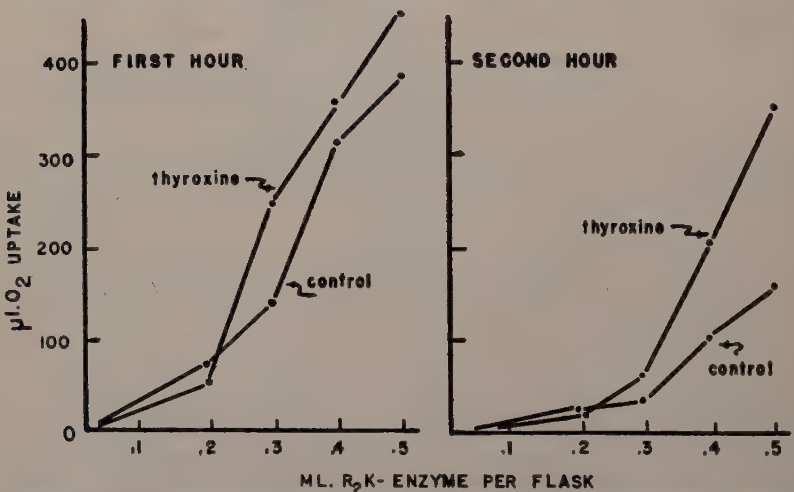


FIGURE 4. Effect of thyroxine on the oxidation of β-hydroxybutyrate by various concentrations of enzyme. The twice washed residue of rat kidney homogenate contained 2.3 mg. N per ml. Up to 0.5 ml. were used per flask. In addition to 15 μ moles of β-hydroxybutyrate, each flask contained 0.9 μ moles of fumarate. Thyroxine was added to give a final concentration of 1.5×10^{-5} M.

it was of interest to study the effects of the hormone on fatty acid oxidation by the washed kidney residue.

As shown in FIGURE 4, thyroxine at 1.3×10^{-5} M inhibited the oxidation of β-hydroxybutyrate at low enzyme concentration but stimulated oxida-

tion when more of the kidney residue preparation was used. The oxidation-enhancing effect of thyroxine was more pronounced during the second hour of the experiment (FIGURE 4). In systems where the washed kidney residue is oxidizing β-hydroxybutyrate in the presence of a limited amount of 4-carbon dicarboxylic acid, appreciable quantities of acetoacetate accumulate. The enhanced oxygen consumption in the presence of thyroxine is accompanied by a decreased accumulation of acetoacetate (FIGURE 5). Incidentally, it may be pointed out that the oxygen consumption data

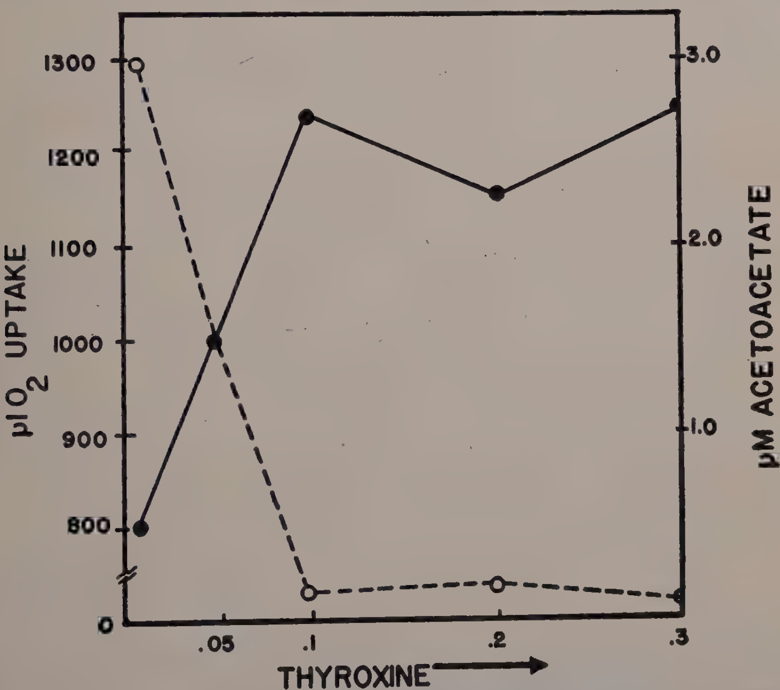


FIGURE 5. Influence of thyroxine concentration on the oxidation of β-hydroxybutyrate and the accumulation of acetoacetate. Ordinate = ml. of 4×10^{-4} M thyroxine added per flask. Each flask contained 1.5 mg. enzyme N, 15 μ moles DL-β-hydroxybutyrate and 0.9 μ moles fumarate. Duration of experiment, 3 hours. The experimental points for both oxygen consumption and acetoacetate accumulation in the presence of 6.5×10^{-6} M thyroxine are superimposed.

show that the kidney preparation is apparently able to oxidize the unnatural *D* isomer of β-hydroxybutyrate.

Similar effects of thyroxine have been obtained with acetoacetate, caproate, or caprylate as substrates. Results of some typical experiments are shown in TABLE 8. The effects of thyroxine were usually greatest during the second hour of the experiment. In several experiments in which the control rate of caprylate oxidation was low, thyroxine had an immediate effect and enhanced oxygen uptake two- to eightfold. Experiment 6, TABLE 8 is representative. The factors which limit the control rate under such conditions have not yet been determined.

The ability of thyroxine to stimulate fat oxidation is apparently specific

for the natural hormone. None of the analogs tested stimulated fatty acid oxidation at concentrations where thyroxine was effective.

Effect of Thyroxine on Enzymatic Phosphorylations. We have presented previously[20] the hypothesis that not only thyroxine, but possibly other hormones, may exert their metabolic effects by "uncoupling" a specific oxidative phosphorylation occurring at a rate-limiting step. The loss in metabolic efficiency which would result from such a process might be more than offset by the enhanced rate of oxidation so that, during a given time interval, more total utilizable energy would be produced in the cell. The hypothesis has been tested with a variety of hormones. Thyroxine had no effect on P/O ratios when added *in vitro* to systems in which the whole Krebs cycle was operating in fresh mitochondria.

TABLE 8

EFFECT OF THYROXINE ON OXIDATION OF FATTY ACIDS AND ACETOACETIC ACID

Exp. no.	*Substrate*	*Enzyme conc. mg. N/flask*	Q_{O_2} (N) control			Q_{O_2} (N) 1.3×10^{-5} M thyroxine		
			1st hr	*2nd hr*	*3rd hr*	*1st hr*	*2nd hr*	*3rd hr*
1	Acetoacetate	1.1	446	192	23	420	372	126
		1.37	404	111	20	427	278	77
2	Acetoacetate	.49	212	49	12	382	129	18
		.74	398	133	12	417	294	96
3	Caproate	.89	211	27		302	172	
		1.48	306	46		324	218	
4	Caprylate	ca 1.	265	17		313	54	
						281*	50*	
5	Caprylate	ca 1.	271	154		286	244	
						281*	231*	
6	Caprylate	ca 1.	105			295		
						355*		

0.9 μ moles of fumarate added to each flask as a sparker. Substrates were added as follows: acetoacetate 30 μ moles; caproate 5 μ moles; and caprylate 3 μ moles per flask.
* Thyroxine concentration 2.6×10^{-5} M.

A study was next made of oxidative phosphorylation in liver preparations from normal and hyperthyroid rats. The latter had been fed a diet containing 0.25 per cent desiccated thyroid for at least two weeks. The rate of oxygen consumption (per mg. N in the liver residue) with each of the 4 substrates tested (TABLE 9) was significantly greater with the hyperthyroid tissue. In both the presence and the absence of fluoride, lower P/O ratios were obtained with hyperthyroid tissue than with normal tissue (TABLE 9). In the presence of fluoride, greater net quantities of phosphate were fixed by the hyperthyroid tissue. The greatly enhanced oxygen consumption, however, brings the calculated efficiency of the oxidative phosphorylation to a lower value than is obtained with the control tissue preparation. The results with these *in vitro* systems are therefore consistent with the hypothesis[20] but of course do not prove it.

More recently, it has been found that thyroxine added *in vitro* depresses

the P/O ratios of tissue preparations which are oxidizing glutamate to succinate. In these systems, malonate is employed to prevent the oxidation of succinic acid. This depression of phosphate fixation is detectable when levels of thyroxine are used which have no significant effect on oxygen consumption. The most striking effects on P/O ratios occur when sufficient thyroxine is used to depress respiration. Under these conditions, the phosphate esterification is relatively more sensitive to thyroxine than is respiration. These results will be presented elsewhere.

Summary. Thyroxine at 10^{-5} M inhibited the oxidation of glutamate by a washed residue of rat kidney homogenized in isotonic KCl. The inhibitory effect is limited to those isomers and analogs of thyroxine which have thyroxine-like activity *in vivo*.

Thyroxine inhibited the oxidation of β-hydroxybutyrate and certain fatty

TABLE 9

OXIDATIVE PHOSPHORYLATION IN WASHED LIVER RESIDUE FROM NORMAL AND HYPERTHYROID RATS

Substrate	*No. of exp.*	*Fluoride added*	*Control*			*Hyperthyroid*		
			O_2/mg. N	*P fixed/ mg. N*	*P/O*	*O_2/mg. N*	*P fixed/ mg. N*	*P/O*
		M	*µ moles*	*µ moles*		*µ moles*	*µ moles*	
Pyruvate..........	6	.01	.83	4.7	2.8	1.08	5.1	2.4
		.00	1.08	2.9	1.3	1.33	2.8	1.1
α-Ketoglutarate....	6	.01	1.21	6.6	2.7	1.48	6.9	2.3
		.00	1.36	3.0	1.1	1.48	2.4	0.8
Glutamate.........	3	.01	.88	5.2	3.0	1.23	5.4	2.2
		.00	.92	1.1	0.6	1.25	1.6	0.6
Succinate..........	3	.01	1.33	3.4	1.3	1.67	4.1	1.3
		.00	1.74	1.4	0.4	2.0	1.0	0.3

3.5–4.5 mg. of nitrogen from washed residue of rat liver homogenate per flask.
Experimental period 10 min. at 30°.

acids at very low enzyme concentrations but greatly enhanced oxidation of these substrates when higher levels of enzyme were employed.

Enzyme preparations from hyperthyroid rats' liver were less efficient in coupling phosphorylation with oxidation than were similar preparations from normal rats. Thyroxine, added *in vitro* to a system oxidizing glutamate, depressed oxidative phosphorylation.

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THE EFFECT OF INSULIN AND PITUITARY HORMONES ON GLUCOSE UPTAKE IN MUSCLE*

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The purpose of this paper is to summarize the present state of our knowledge about two problems: first, the means by which insulin enhances glucose uptake by tissues; and second, the nature and loci of action of the anterior pituitary fractions which influence glucose uptake and utilization. Experiments with whole or eviscerated animals, with isolated tissues, and with cell-free enzyme systems will be considered. Because of limitations of time and space, I shall discuss principally experimental work by our own group* and will not undertake a complete literature review.

I. INSULIN AND PITUITARY EFFECTS ON GLUCOSE UPTAKE AND UTILIZATION

A. Whole and Eviscerated Animals. *1. Insulin.* Knowlton and Starling[36] found that the glucose use of the dog heart-lung preparation, normally about 4 mg. per gm. of heart per hour, was reduced nearly to zero by previous pancreatectomy. The preparation had an R. Q. of 0.7. The reduced rate of glucose uptake and low R. Q. could be restored to normal by using normal blood as perfusing fluid or by adding to the diabetic blood an acid extract of pancreas. These remarkably prescient observations were confirmed and extended much later by Cruickshank and Startup,[16] after it became possible to substitute insulin for crude extracts of pancreas. Pancreatectomy likewise lowered glucose use by the eviscerated cat.[6]

In diabetic rats, injected with C^{14} glucose at a constant rate, the amount of C^{14} glucose disappearing and the fraction transformed to CO_2 were reduced, as compared to the normal values.[69] This direct demonstration of low CO_2 production substantiates earlier measurements[60] showing that muscle strips from depancreatized dogs had an R. Q. of 0.73 as compared to the normal of 0.94.

In the converse type of experiment, insulin added to the perfusion fluid was found to raise glucose uptake from 0.38 to 0.67 mg. per gm. cat hind leg per hour,[3] and this result has been confirmed.[47]

The action of insulin upon glucose uptake is a specific one. Cori and Cori[13] determined the effect of insulin upon use of glucose, fructose, or mannose by the eviscerated rat (table 1). Insulin stimulated specifically the removal of glucose, but not that of fructose or mannose. Lundsgaard and collaborators[22] found similarly that, while the use of glucose by the perfused cat hind leg was increased by insulin, the use of fructose was not.

Muscle contains separate enzymes for phosphorylation of glucose and fructose.[61] Insulin apparently acts only on the glucokinase. The insulin effect must be at a point before the metabolic pathways of glucose and

* The unpublished experimental work presented in this summary has been performed largely by Drs. Charles R. Park, David H. Brown, Marvin Cornblath, William H. Daughaday, and Lillian Recant. The author wishes to thank them for making these observations available for this monograph. The author also wishes to express his appreciation to Dr. Carl F. Cori for his advice and encouragement.

fructose are joined, and this leaves practically only the first reaction, phosphorylation of glucose by ATP, as the point of action of insulin (FIGURE 1).

It has been reported that removal of galactose from the circulating blood

TABLE 1

EFFECT OF INSULIN ON UTILIZATION OF VARIOUS SUGARS BY EVISCERATED RATS*

Sugar injected	Sugar recovered after injection (per cent of total injected)		
	5 minutes	60 minutes	60 minutes plus insulin
Glucose	97	90	2
Fructose	98	61	62
Mannose	91	61	54

* Known amounts of glucose, fructose, or mannose were injected intravenously without and with added insulin and the entire carcass was analyzed for free sugar at 5 or 60 minutes after injection (from Cori and Cori[13]).

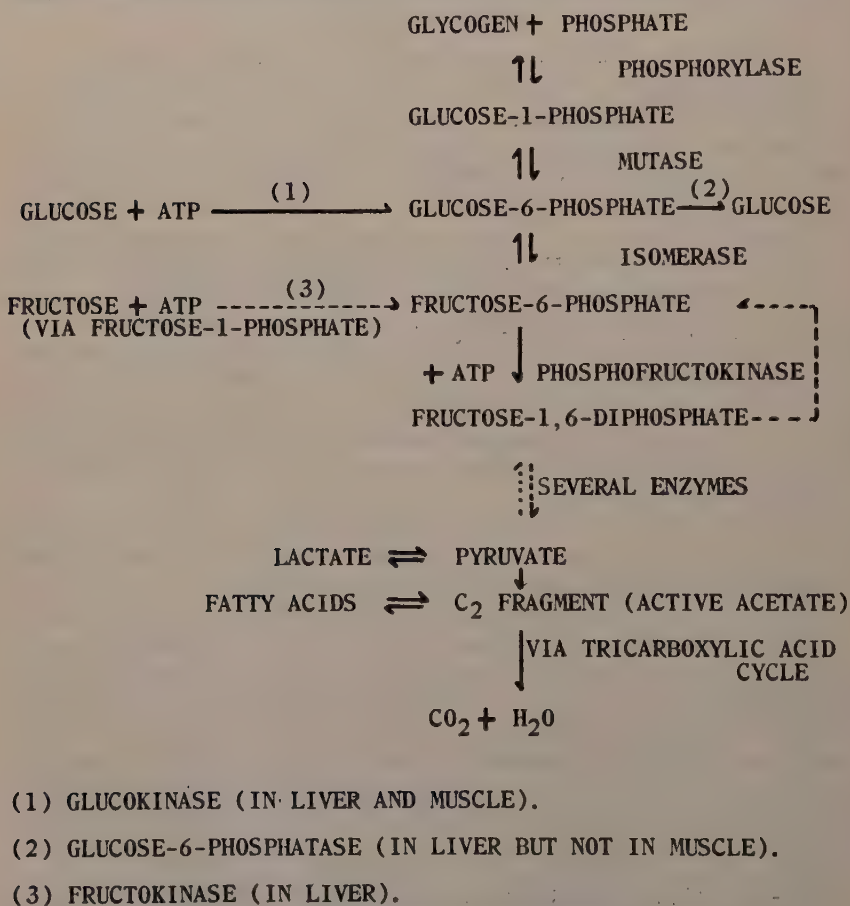

FIGURE 1. Some intermediate reactions for glucose and fructose use by muscle and liver.

of the eviscerated dog is stimulated by insulin.[41] This result cannot yet be related to those with glucose and fructose, as the metabolic fate of galactose in muscle is not known.

2. *Pituitary*. Houssay[29-32] provided the initial lead for investigation of the relation of the anterior pituitary to carbohydrate utilization in his finding that diabetes was ameliorated after hypophysectomy and intensified by administration of crude anterior pituitary extracts to depancreatized-hypophysectomized animals. The fact that pituitary extracts can accentuate diabetes in the animal devoid of its pancreas shows that the pituitary effect is not merely one of preventing insulin from reaching its site of action. Lukens has found that Houssay dogs exhibit an increased glycosuria without change in nitrogen excretion after administration of crystalline growth hormone.[44] Highly purified pituitary products, including growth and adrenocorticotropic fractions, also accentuated glycosuria in partially diabetic rats.[2] Conversely, the proportion of carbohydrate used in the metabolism of the intact or eviscerated hypophysectomized animal is higher than that in the corresponding normal.[18, 19, 58]

3. *Adrenal Cortex*. The role of the adrenal cortex in glucose utilization of the whole animal was emphasized in the experiments of Long and Lukens.[43] The rate of glucose disappearance in eviscerated adrenalectomized rats was higher than that for the eviscerated normal,[34] and injection of C-11 oxysteroids into eviscerated normal or eviscerated adrenalectomized rats reduced glucose uptake.[35]

These experiments on whole or eviscerated animals suggest very strongly that glucose use is decreased in insulin deficiency and enhanced by insulin excess, and that the reverse obtains with respect to the secretions of the anterior pituitary and adrenal cortex. There are obvious difficulties in a study of the mechanism of action of these substances in whole or eviscerated animals. It was, therefore, of considerable advantage that an action of these substances could also be demonstrated in isolated tissues, in some cases by adding them *in vitro* and in others by injecting the animal prior to removal of the tissue.

B. Excised Tissues. It is now believed that glucose use by tissue passes through the sequence of reactions shown in figure 1. The hexokinase (glucokinase) reaction, being the first step in the series and being irreversible, is the one which limits glucose uptake in muscle extracts. The question has been raised as to whether glucose uptake can be used as a measure or the rate of the hexokinase reaction in intact muscle. The first requirement for the use of glucose uptake in this sense is that ATP is not limiting. That ATP is not a limiting factor in the present experiments is shown by the following three examples: (a) The ATP level in normal muscle is many times that required to saturate hexokinase;[61] (b) the hexokinase content of rat diaphragm muscle does not vary significantly from the normal when glucose uptake is reduced in diabetes or enhanced by excess insulin, the values for the easily hydrolyzable P of ATP being 18 mg. per cent for diabetic diaphragms with reduced glucose uptake[37] and 17–22 mg. per cent for normal diaphragms[37, 74]; and (c) even under anaerobic conditions, where the acid-labile P concentration is only 0.03 mg. per cent, the glucose up-

take of diaphragm can be raised from 2.3 mg. per gm. per hour to 4.2 by increasing the glucose concentration from 140 mg. per cent to 420 mg. per cent.[74]

The second condition for equating rate of glucose uptake and rate of the hexokinase reaction in muscle is that the glucose transformed to glucose-6-phosphate does not appear again as glucose. The hexokinase reaction itself is virtually irreversible because of the large energy drop from ATP to glucose phosphate. Indirect liberation of glucose from glycogen or glucose phosphate would not be expected, because of absence of amylase or phosphatase activity in the intact muscle. That glucose liberation, in fact, does not occur in the intact muscle is demonstrated by the finding that radioactive C^{14} added to excised diaphragms as carbonyl labelled pyruvate can be transformed to glycogen but not to glucose.[73]

From the scheme of FIGURE 1, it is apparent that glucose utilization may be measured directly, as we have chosen to do, because of the possible relation of glucose disappearance to hexokinase activity, or estimated indirectly as one of the products which the tissue forms from glucose-6-phosphate: glycogen,[64] lactic acid,[10] or carbon dioxide.[78] FIGURE 1 will make it clear that any hormonal effect on the rate of formation of one of these products from glucose cannot be separated from an effect on the initial reaction of the series, *i. e.*, the phosphorylation of glucose, until an effect on some subsequent step is specifically demonstrated.

1. Glucose Utilization by Rat Diaphragm. Gemmill[23] showed that glucose use and glycogen formation by the excised rat diaphragm are enhanced by addition of insulin to the medium *in vitro*, an observation later confirmed by many investigators.* The effect was produced by amorphous insulin, by zinc-insulin crystals, or by insulin free of hyperglycemic factor. It was not produced by insulin which had had its hypoglycemic activity destroyed in various ways and was therefore a specific effect of insulin itself. Of the glucose taken up by the diaphragm of the normal rat, Gemmill[24, 25] recovered about 60 per cent as acid hydrolysable polysaccharide. Walaas and Walaas[75] recovered 50 per cent of the glucose used by normal diaphragm as glycogen, and a similar proportion was obtained under a number of other experimental conditions (TABLE 2). Walaas and Walaas also re-

* The procedure used by Gemmill, which has been adopted in principle by subsequent investigators, was to remove the diaphragms, blot lightly to remove excess blood, and transfer to a physiological salt solution. Samples of the diaphragm and medium are analyzed at the beginning and at the end of the experimental period for glucose, lactic acid, or other pertinent substances. If initial values are high, as is the case for lactic acid in normal or epinephrine-injected rats,[25] or for glucose in severely diabetic rats,[38] the diaphragms must be equilibrated with the chilled incubation medium prior to use.

When measured over a 30-minute period at 37° C., the rate of glucose uptake depends on the extent to which the diaphragms have been preincubated in chilled Krebs-bicarbonate medium.[51] The rates of glucose uptake increase sharply with preincubation times up to five minutes and tend to reach a constant rate after 15–30 minutes exposure to the chilled medium. The values given in this paper were obtained with approximately 15 minutes pre-exposure to chilled medium. With shorter pre-exposure times, the absolute rates are lower for diaphragms from normal and hypophysectomized rats and from hypophysectomized rats injected with growth hormone, but the effects of hypophysectomy and growth hormone injection are qualitatively the same as those here described. Li, Kalman, and Evans,[42] who found no increase in glucose uptake after hypophysectomy (in contrast to Krahl and Park, and Villee and Hastings), do not state whether and to what extent each group of diaphragms was preincubated in chilled medium. In investigating effects of hormones on diaphragm metabolism, careful attention must be given to other conditions of the experiment. The strain of the rat and its dietary history prior to removal of diaphragms must also be rigidly controlled. At the time of removal, the diaphragm carries with it a supply of insulin which depends on the level of islet secretory activity immediately preceding. Glucose uptake is, therefore, higher in diaphragms from fed than from fasted rats. Similar consideration must also be given to the state of secretory activity of the pituitary, the adrenals, and perhaps the thyroid.[62]

covered 25 per cent of the glucose used as lactic acid. Another 5–10 per cent of glucose disappearing from the medium can be recovered from C^{14} labelled glucose as respiratory carbon dioxide.[1, 72] Thus, some 80 per cent of the glucose used is accounted for.[73, 75]

The glucose uptake by diaphragm is very reproducible under any given set of experimental conditions. The rate is higher with a 30-minute than with a 60- or 120-minute incubation period (TABLE 3). It is higher at 300 or 500 mg. per cent glucose concentrations than at 200 mg. per cent or less, and, with one exception,[28] all published measurements indicate that insulin produces an enhancement of glucose uptake or glycogen synthesis at glucose concentrations up to 500 mg. per cent.[23, 72, 73]

Table 2

Relation of Glycogen Formation to Glucose Uptake under Various Experimental Conditions. One Hour Period of Incubation

Preparation of rat and reference	*Injection*	*Insulin content of incubation medium*	*Glucose uptake mg. per gm. wet tissue per hour*	*Glycogen formed as per cent of glucose uptake*
		units/ml.		
Normal[75]	None (16 rats)	0	2.8	50
Hypophysectomized[50]	None (18 rats)	0 0.1	4.6 5.9	49 53
	Growth hormone, 400 μg. 24 hours, before use of diaphragm (6 rats)	0 0.1	3.3 5.0	53 58
Hypophysectomized—adrenalectomized[50]	None (12 rats)	0 0.1	4.0 5.7	49 51
	Growth hormone, 400 μg. 24 hours, and lipo-ACE, 0.2 ml. at 24 and 3 hours before use (12 rats)	0 0.1	3.2 5.7	48 57

Insulin stimulates not only glucose use, but, as would be expected from an increased rate of formation of glucose-6-phosphate, also the deposition of glycogen[23, 64, 72] and the formation of carbon dioxide[72, 78] from labelled glucose.

The formation of glycogen in diaphragm from C^{14} labelled pyruvate is not enhanced by insulin.[73] Thus, on the basis of the scheme presented in FIGURE 1, the effect of insulin on diaphragm appears to be acceleration of the interaction of glucose with ATP.*

Glucose use by diaphragms from severely diabetic (alloxan) rats is only

* The lack of a significant acceleration by insulin of glycogen formation from pyruvate is of some importance in relation to the suggestion that the action of insulin is to increase the amount of ATP available for synthetic reactions rather than to facilitate specifically interaction of glucose with ATP.[26, 53, 63] Transformation of two pyruvate molecules to one hexose residue of glycogen requires four "high energy" phosphate groups from aerobic metabolic sources, whereas formation of one glucose residue in glycogen from glucose requires only one. Hence, an increase in available ATP might be expected to accelerate pyruvate conversion to glycogen as well as glucose conversion to glycogen. This is not found experimentally.

TABLE 3

COMPARISON OF GLUCOSE UPTAKES BY RAT DIAPHRAGMS AS MEASURED BY DIFFERENT TECHNIQUES AT 37–38°C.*

Method	Glucose uptake, mg. per gm. wet tissue per hour: Normal		Hypophysectomized		Hypophysectomized-adrenalectomized	
	in glucose	*in glucose + insulin*	*in glucose*	*in glucose + insulin*	*in glucose*	*in glucose + insulin*
I	1.8	3.4				
II	1.9	3.5				
III	1.6 ± 0.06†	2.3 ± 0.15	2.5 ± 0.08	3.3 ± 0.13		
IV	1.5 ± 0.05	2.9 ± 0.05	2.6 ± 0.05	4.4 ± 0.08	3.3 ± 0.05	6.1 ± 0.07
V(A)	2.7 ± 0.19	4.5 ± 0.28	4.3 ± 0.26	5.6 ± 0.27		
V(B)			3.8 ± 0.10	5.5 ± 0.10	3.1 ± 0.09	5.9 ± 0.25
VI	3.6 ± 0.11	6.6 ± 0.15	4.9 ± 0.09	6.8 ± 0.14	4.7 ± 0.17	7.1 ± 0.25

* Method I (Gemmill and Hamman[25]), 3-hour period of incubation, 200 mg. per cent initial glucose; II (Stadie[63]), 2 hours, 200 mg. per cent glucose; III (Krahl and Cori[33]), 2 hours, 100 mg. per cent glucose: IV (Villee and Hastings[72]), 2 hours, 200 mg. per cent glucose; V (A) (Krahl and Park[39]), 1 hour, 140 mg. per cent glucose; V (B) (Park and Daughaday[50]), same conditions as V (A); VI (Park and Daughaday[50]), one-half hour, 140 mg. per cent glucose.
† Standard error.

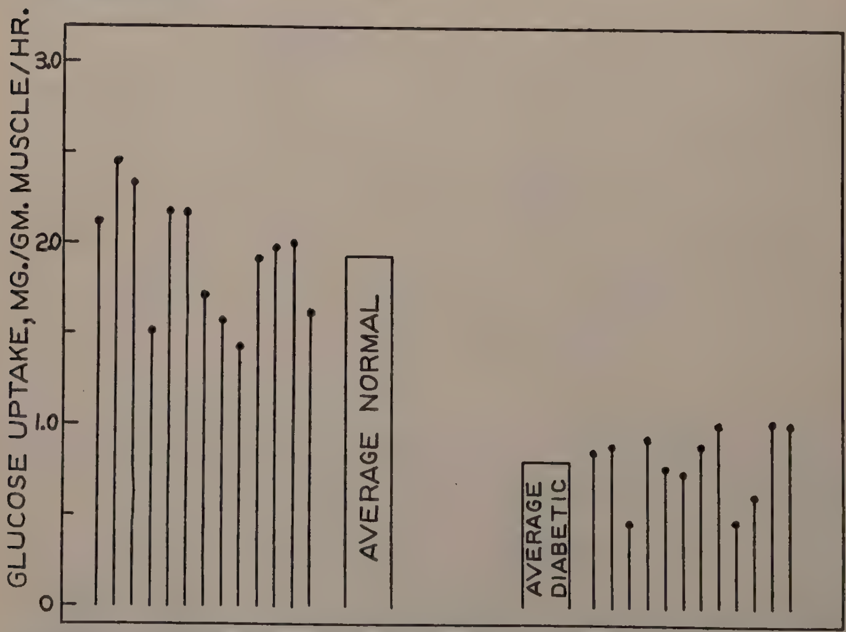

FIGURE 2. Glucose uptake by diaphragms from normal and severely diabetic (alloxan) rats. The muscle was shaken in oxygenated Krebs-bicarbonate solution and the medium analyzed for glucose before and after two hours of incubation at 37°C. The initial glucose concentration of the medium was 100 mg. per cent.

50–60 per cent of normal (FIGURE 2).[38, 72*] Glycogen and CO_2 production are also decreased.[72] Added insulin raises the rate of glucose use toward normal (TABLE 4). Krahl and Cori[38] emphasized that diaphragms with

* Corboz[12] measured glucose uptake of normal and diabetic diaphragms at pH 6.2 where, in contrast to the results obtained at pH 7.4 by all other workers, there was no glycogen synthesis without or with insulin.

low glucose use could be obtained only from diabetic rats with fasting blood sugars above 300 mg. per cent.

When the adrenals are removed from diabetic animals, there is an amelioration of the signs of diabetes, as had been discovered by Long and Lukens.[43] In TABLE 5 are shown the average blood sugar values of the rats whose diaphragms were used for measurement of glucose uptake. The blood sugar returns to normal when diabetic-adrenalectomized rats are

TABLE 4

GLUCOSE UPTAKE BY DIAPHRAGMS FROM DIABETIC, FROM DIABETIC-ADRENALECTOMIZED, AND FROM ADRENALECTOMIZED RATS*

Investigators	Conc. glucose in medium	Glucose uptake, mg. per gm. wet tissue per hour							
		Normal		Adrenalectomized		Diabetic		Diabetic-adrenalectomized	
		in glucose	in glucose + insulin	in glucose	in glucose + insulin	in glucose	in glucose + insulin	in glucose	in glucose + insulin
	mg. %								
Krahl and Cori[38]	100	1.8	2.4	2.0	2.4	0.8	1.4	2.2	2.9
Villee and Hastings[72]	200	1.5	2.9	2.3	3.9	0.8	2.2	3.1	5.2

* Measurements were made with a 2-hour incubation period. All diabetic rats had fasting blood sugars of 300 mg. per cent or more.

By statistical analysis, the insulin stimulation is significant in each pair. Also, all operative procedures produce a significant difference from the normal except in the Krahl-Cori adrenalectomized series.

TABLE 5

BLOOD SUGARS (MG.%) OF SEVEN ALLOXAN-DIABETIC RATS BEFORE AND AFTER ADRENALECTOMY*

Before adrenalectomy		After adrenalectomy	
fed	fasting (20 hr.)	fed	fasting (6 hr.)
550	410	330	90

* The diaphragms from these animals were used for the Krahl and Cori[38] experiments on glucose uptake which are recapitulated in TABLE 4.

deprived of food for a short time. Parallel with the decrease in the blood sugar level after adrenalectomy, there is a return of glucose uptake by muscle to normal (TABLE 4). These and other experiments to be presented below suggest that the adrenals exert an inhibitory effect on the hexokinase reaction. This influence is not revealed unless some pituitary factor is also present.

Krahl and Park[39] observed that glucose uptake of diaphragms from hypophysectomized rats is higher than that of the normal, with an optimum 15–20 days after operation (FIGURE 3). Increases in rate following hypophysectomy or removal of both the pituitary and the adrenals have also

been reported by Villee and Hastings[72] and by Park and Daughaday.[50] No increase in glucose uptake following hypophysectomy was observed by Perlmutter and Greep,[52] using animals four days after operation, or by Li, Kalman, and Evans,[42] using animals 10 days after operation. As shown in FIGURE 3, the effect of hypophysectomy on glucose uptake does not become fully established until about 10 days after operation, at which time the adrenal cortex has undergone considerable atrophy.

In view of these regressive changes in the adrenal following hypophysectomy, it is pertinent to inquire what effect upon glucose uptake is produced by adrenalectomy. Krahl and Cori[38] (TABLE 4) found glucose uptake of diaphragms from adrenalectomized rats to be slightly increased. Villee and Hastings[72] observed larger increases. In no case, however, was the

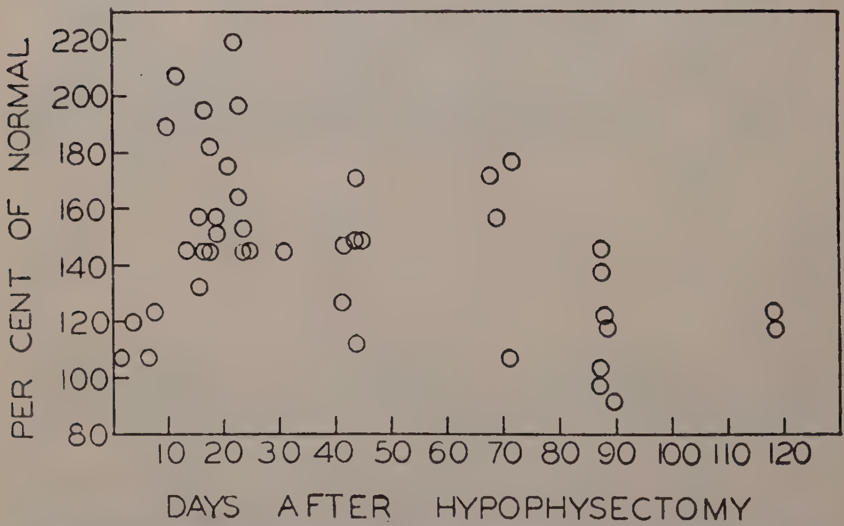

FIGURE 3. The glucose uptake without added insulin of diaphragms from rats at various times after hypophysectomy compared on a percentage basis to average uptake of diaphragms from normal rats.

effect from adrenalectomy alone large enough to account for the increase in glucose uptake which follows hypophysectomy. The results of replacement therapy (see below) show that both a pituitary factor and an adrenal cortical factor are required to reduce to normal the glucose uptake of diaphragms from hypophysectomized-adrenalectomized rats.*

The isolated muscle of the hypophysectomized or hypophysectomized-adrenalectomized rat is still responsive to insulin.[72, 39, 50, 52] Typical results are shown in FIGURE 4. Under these conditions, the level of glucose uptake attained with 0.1 unit per ml. of added insulin is the same with diaphragms from normal, from hypophysectomized, or from hypophysectomized-adrenalectomized rats. The fact that insulin still stimulates glu-

* Glucose uptake and glycogen formation have been studied in diaphragms taken from hypophysectomized rats injected for 11 days with 200 micrograms ACTH per day. No significant change in glucose uptake or glycogen formation was produced. Efforts to test effects of adrenal steroids directly *in vitro* have not yielded decisive results because of the difficulty in obtaining these agents in aqueous solution. Verzar and his collaborators[40, 71] observed depression of both glucose uptake and glycogen formation when solutions of each of a number of steroids in 0.5 per cent ethanol were added directly to diaphragms from normal rats.

cose uptake in diaphragms from hypophysectomized-adrenalectomized rats shows very clearly that the glucose uptake of muscle is in part under an insulin-reversible inhibitory influence from the pituitary and adrenals and in part under an inhibition which persists even when these glands are removed, a conclusion stressed by both Krahl and Park[39] and Perlmutter and Greep.[52] The mechanism of this inhibition is unexplored. The nature of the pituitary-adrenal inhibitors will be discussed later in this paper.

2. Glucose Utilization by Liver Slices. Direct balance experiments on

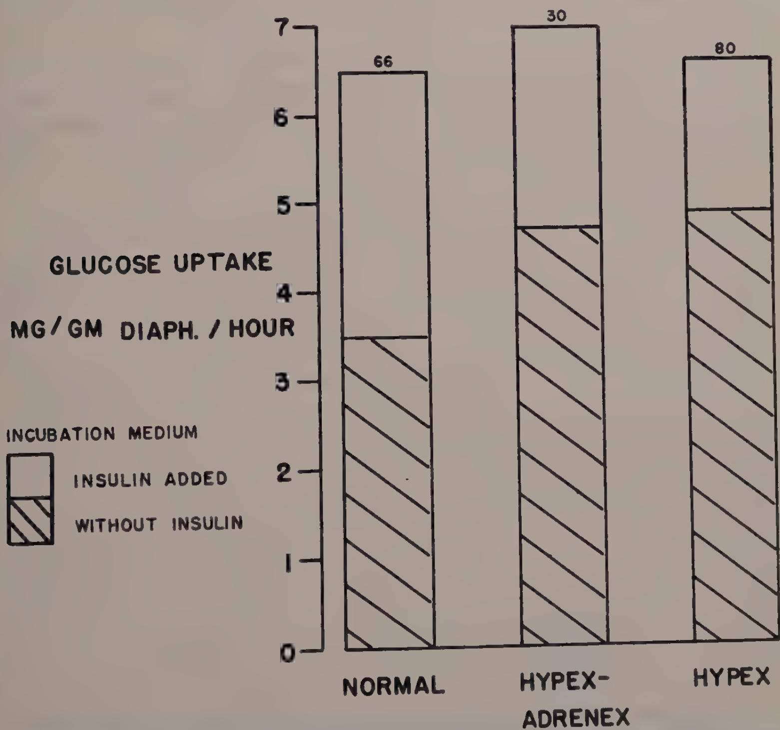

FIGURE 4. Effect of hypophysectomy and hypophysectomy plus adrenalectomy on glucose uptake by rat diaphragm. The height of the cross-hatched area represents the rate without added insulin; the total height represents the rate with 0.1 unit insulin per ml. of incubation fluid. The number of rats used is given at the top of each bar.

uptake and utilization of glucose by liver are not possible, because glucose transformed to glucose-6-phosphate over the hexokinase reaction may be immediately returned to the medium as glucose *via* the glucose-6-phosphatase, which is present in liver but not in muscle (FIGURE 1). These difficulties can now be circumvented in part by use of C^{14} labelled glucose. Teng, Sinex, and Hastings[70] found that rates of formation of glycogen and other constituents of liver (fats, amino acids) from C^{14} labelled glucose by liver slices were decreased in diabetes and increased after adrenalectomy. In the former case, addition of insulin *in vitro* brought glucose utilization back toward normal.

Measurement[8] of the use of glucose and fructose by liver slices from

normal and diabetic rats yielded results which parallel those on muscle[13, 22] in indicating that insulin stimulates glucose use but not fructose use. The conversion of C^{14} glucose to CO_2 was depressed in the diabetic liver, while that of C^{14} fructose to CO_2 was not. The obvious inference is that glucokinase activity is dependent on insulin, while that of fructokinase is not. An effect of insulin on the isomerase step is excluded by the well-known fact that breakdown of glycogen in liver and muscle is not impaired in diabetes.

Another locus for insulin action in liver is indicated by the finding that the conversion of C^{14} acetate to fatty acids in liver slices is lower in diabetic than in normal liver.[4, 8]

3. Glucose Uptake by Adipose Tissue. Since adipose tissue can be readily obtained from various species, including man, for the study of hormone effects, it was of interest to determine whether it can take up glucose directly and whether an effect of insulin on glucose uptake is demonstrable.

TABLE 6
GLUCOSE UPTAKE BY RAT ADIPOSE TISSUE*

Type adipose tissue	*Glucose uptake, mg. per gm. wet tissue per hour*		*Increase with insulin*
	in glucose	*in glucose + insulin*	%
Subcutaneous			
normal (7)†	0.54	0.96	+78
diabetic (4)	0.31	0.59	+90
Genital			
normal (3)	0.68	1.01	+49
diabetic (4)	0.28	0.54	+93
Perinephric (3)	0.86	1.05	+22
Mesenteric (3)	1.40	2.11	+50

* From Krahl.[37]
† The number of rats used is given in parentheses.

Wertheimer and Shapiro[77] had previously emphasized that glycogen can appear in adipose tissue when a severely starved rat is fed carbohydrate, and the Chaikoff group[45] observed that C^{14} glucose could be transformed to fatty acids in the eviscerated rat.

The measurements given in TABLE 6 show that glucose is used by rat adipose tissue and that this use is stimulated by insulin.[37] Similar effects of insulin were obtained with adipose tissue from the normal dog and the diabetic cat.

The interesting observation has been made that the mammalian red cell, in which glucose uptake is normally not increased by insulin, apparently has less hexokinase after production of alloxan diabetes than before.[9]

The experiments cited up to this point show that insulin stimulates directly the glucose uptake of three tissues of major metabolic importance: striated muscle, liver, and adipose tissue. They show that, in the case of striated muscle, part of the insulin-reversible inhibition is produced by products of the pituitary and adrenal glands and part by factors which are present long after removal of these glands.

The experiments also indicate that insulin accelerates the conversion of glucose to carbon dioxide and glycogen, but not that of fructose to carbon dioxide or of pyruvate to glycogen. This clearly points to the glucokinase reaction as one locus of action of insulin.

An effort was made by Colowick, Cori, and Slein[11] to test directly the hypothesis that one action of insulin consists in reactivating glucokinase previously subjected to inhibition by tissue or hormonal factors. They found that, under very precisely specified conditions, glucose use by extracts of diabetic muscle could be stimulated by insulin in about half their experimental trials. Reid, Smith, and Young[55] reported positive insulin effects in a smaller proportion of trials with a similar system. Stadie and Haugaard,[67] using a more vigorous extraction procedure than that specified by the original investigators, obtained completely negative results. Colowick, Cori, and Slein had already reported that the procedure used by the Stadie group was unsuitable for *in vitro* test of insulin effects. Weil-Malherbe[76] found in the blood of untreated human diabetics an inhibitor for brain hexokinase. This inhibitor effect decreased markedly after the patient was treated with insulin.

Colowick, Cori, and Slein also tested the effect of pituitary and adrenal extracts upon brain hexokinase and obtained insulin-reversible inhibitions in a certain proportion of their trials. Similar results were reported by Broh-Kahn and Mirsky.[5] Reiss and Rees[56] found the hexokinase activity extractable from rat brain to be higher after hypophysectomy than before.

On the whole, it has been difficult to obtain, with a cell-free enzyme system, consistent responses to variation in hormonal balance, a fact stressed in the original report. We therefore set out, some years ago, to study in detail the individual components of the extract system, the objective being to learn enough about the hexokinase system on the one hand and the pituitary-adrenal inhibitors on the other to make it possible ultimately to set up a system which would be completely reproducible. The rest of the present discussion will deal with efforts to obtain a pituitary inhibitor of glucose uptake in a stable form and to characterize it from a chemical standpoint.

II. NATURE OF PITUITARY FACTORS INFLUENCING GLUCOSE UPTAKE AND UTILIZATION

A. Glucose Uptake. The principal difficulty encountered in the extract work was the instability of both the test system and the inhibitor. Since the glucose uptake of the rat diaphragm under a fixed set of experimental conditions had proved to be very reproducible, it was decided to use diaphragms in an assay system for purification of the pituitary inhibitor of glucose uptake.

1. Inhibition of Glucose Uptake by Highly Purified Growth Hormone. Experiments have been made with Armour growth hormone lot no. 22KR2 and with electrophoretically homogeneous samples of growth hormone which we have prepared by one to four crystallizations, according to a slight modification of the method of Wilhelmi, Fishman, and Russell.[79] The growth hormone was injected intraperitoneally into hypophysectomized

or hypophysectomized-adrenalectomized rats 18–24 hours before removal of the diaphragm. The lipo-adrenal extract was the commercial Upjohn product and was injected intramuscularly in divided doses 24 and six hours before testing. Cortisone gave, in this system, effects qualitatively similar to the lipo-adrenal extract. These experiments were carried out with a 30-minute period of incubation, in which the control rates are somewhat higher than for the 60-minute period (TABLE 3).

In diaphragms from hypophysectomized animals, growth hormone in

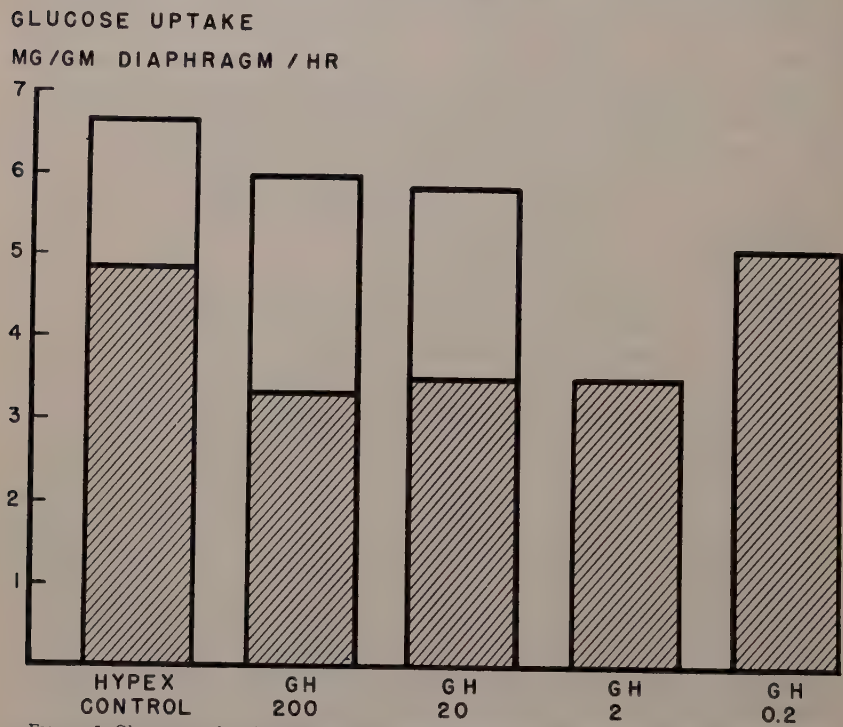

FIGURE 5. Glucose uptake of diaphragm from hypophysectomized rats 24 hours after intraperitoneal injection of various amounts of growth hormone (GH); the number under each bar is the dose in micrograms. For significance of cross-hatched and white areas, see FIGURE 4.

dosages of 200, 20, or 2 micrograms per 100 grams of rat produced a highly significant depression in all cases; 0.2 microgram was ineffective (FIGURE 5). Two samples of growth hormone prepared by different procedures were equivalent in their ability to depress glucose uptake and to stimulate growth when administered to hypophysectomized rats over a four-day period prior to removal of diaphragms and measurement of the width of the tibial epiphyseal cartilage (TABLE 7).

Two conclusions emerge from these measurements: first, as the dose of two micrograms of growth hormone is nearly a minimal one to produce significant growth effects in these rats, it is clear that growth hormone may very well play a physiological role in control of glucose uptake by muscle; second, in the presence of 0.1 unit per ml. of added insulin, the rates after

growth hormone injection into the hypopysectomized rats were not significantly different from those for the control diaphragms in the same concentrations of insulin. The inhibition by this dosage of growth hormone is, therefore, completely reversed by insulin, *if the insulin concentration is high enough.* We have observed that 10^{-4} units of insulin per ml. cannot reverse the growth hormone inhibition completely, especially when repeated

TABLE 7

COMPARISON OF EFFECTS OF TWO SAMPLES OF GROWTH HORMONE ON GLUCOSE UPTAKE OF DIAPHRAGM AND BONE GROWTH. FOUR DAILY INTRAPERITONEAL INJECTIONS WERE GIVEN TO 100 GM. MALE HYPOPHYSECTOMIZED RATS, FOLLOWED BY A 24-HOUR FASTING PERIOD PRIOR TO DIAPHRAGM REMOVAL*

Growth preparation injected	*Dose per day*	*Change in glucose uptake by diaphragm, mg. per gm. wet tissue per hr.*	*Width tibial epiphyseal cartilage*
	micrograms		*microns*
Armour 22KR2	0 (6)†	0	125
	2.1 (7)	−0.6	147
	22 (5)	−1.5	227
Four times recrystallized by Wilhelmi method	0 (14)	0	124
	2.3 (15)	−1.1	157
	23 (16)	−1.6	219

* From Park, Brown, Cornblath, and Daughaday.[51]
† The number of rats used at each dose is given in parentheses.

TABLE 8

EFFECT OF 10^{-4} AND 10^{-1} UNITS OF INSULIN PER ML. ON GLUCOSE UPTAKE BY DIAPHRAGMS TAKEN FROM HYPOPHYSECTOMIZED RATS AFTER INJECTION OF ARMOUR GROWTH HORMONE 22KR2*

Dose growth hormone	*Glucose uptake, mg. per gm. per hour*		
	in glucose	*in glucose + 10^{-4} unit insulin per ml.*	*in glucose + 10^{-1} unit insulin per ml.*
None—controls	4.9	5.7	6.8
400 μg. 24 hours before diaphragm use	3.9	4.7	6.6
5 μg/day for 10 days	2.7	3.5	5.6
10 μg/day for 10 days	3.5	3.7	5.1
20 μg/day for 10 days	2.6	3.1	5.3

* From Park, Daughaday, Brown, and Cornblath.[51]

doses of growth hormone are given (TABLE 8[51]). This gives rise to the suggestion that there may be some sort of mass action effect between the pituitary factor and insulin.

In diaphragms from hypophysectomized-adrenalectomized rats, growth hormone alone produced a slight depression of glucose uptake. The effect is greatly intensified by doses of lipo-adrenal extract, which alone have no effect (FIGURE 6). This shows that both the growth hormone and adrenal cortical factors are involved in the inhibition of glucose uptake by muscle. The inhibition is completely reversed by 0.1 unit of insulin per ml.

2. Inhibition of Glucose Uptake by Crude Pituitary Fractions. In the experiments just described, the highly purified growth hormone was always injected 18–24 hours before diaphragm removal. When 10–400 micrograms of the Armour 22KR2 or of the twice-crystallized samples were injected 3–6 hours prior to diaphragm removal, no consistent inhibition of glucose uptake resulted. Neither 400 micrograms of ACTH (Armour lot no. 57A)

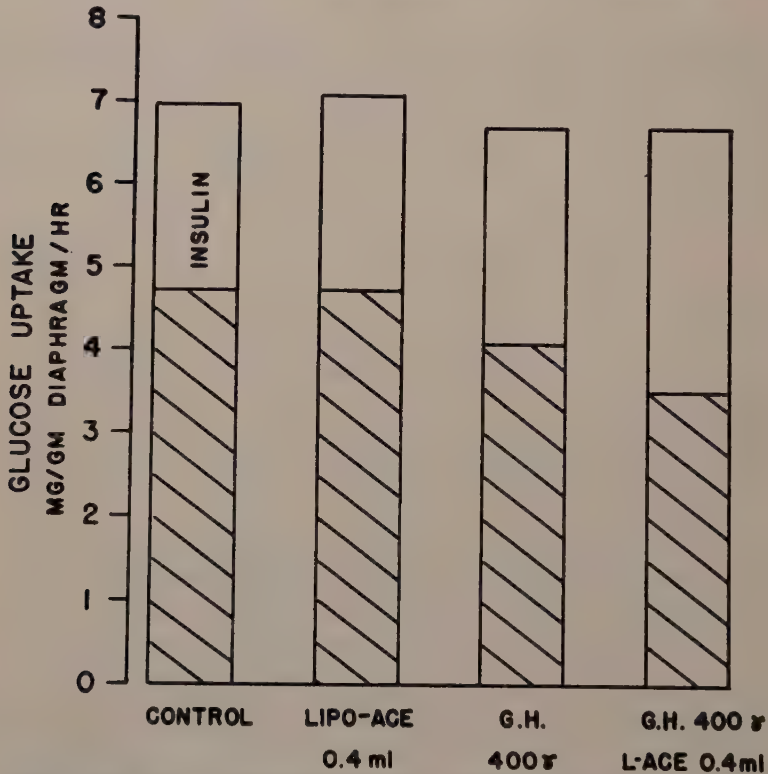

FIGURE 6. Glucose uptake of diaphragms from hypophysectomized-adrenalectomized rats. The growth hormone was given intraperitoneally 24 hours, the lipo-adrenal extract in divided doses at 24 and 6 hours, prior to removal of diaphragms. For significance of cross-hatched and white areas, see FIGURE 4.

alone nor 400 micrograms ACTH plus 400 micrograms growth sample 22KR2 was active under these test conditions.[60]

In earlier exploratory experiments, Park and Krahl[49] found that depression of glucose uptake of diaphragms from normal or hypophysectomized rats could be produced by doses of 10 mg. of Wilhelmi Fraction A or 3 mg. once-crystallized growth hormone given 3–6 hours prior to diaphragm removal. Smaller amounts were not tested. In later experiments by Park and Daughaday,[60] depression of glucose uptake in the 3-hour test were observed with as little as 10 micrograms of Fraction A or Fraction A-1 (an isoelectric precipitate at pH 5 from Fraction A, see TABLE 9).

Since crystalline pituitary proteins with high growth activity are inactive toward glucose uptake 3 hours after injection but inhibiting at doses of

2–20 micrograms if injected 24 hours prior to diaphragm removal, the suggestion arises that an entity which is fully active for growth but inactive toward glucose uptake may be transformed in the body to an active inhibitor of glucose uptake. The rapid acting form of the inhibitor appears to be present in crude pituitary extracts and to be reduced in concentration with repeated crystallizations or precipitations of the growth hormone as they are now carried out. Another complicating factor (see below) is the insulin-like activity of certain growth hormone samples which may obscure short-term inhibitor activity of growth fractions.

Stadie and his coworkers[65, 66, 68] measured glycogen deposited in the rat diaphragm after pre-exposure to pituitary factors and insulin. Two hemi-diaphragms were incubated briefly in solutions of pituitary extract, and

Table 9

Effect of Injection of Growth Hormone Fractions upon Glucose Uptake by Diaphragms from Normal and Hypophysectomized Rats*

Investigators	*Type of rat*	*Pituitary fraction injected and dose, as wt. protein per 100 gm. rat*	*Hours after injection*	*No. of rats*	*Glucose uptake, mg. wet tissue per hr.*	
					in glucose	*in glucose + insulin*
Park and Krahl[49]	normal	none	—	6	3.0	4.2
		3 mg. crystalline growth I	4	6	1.8	3.5
	hypophysectomized	none	—	49	3.8	5.5
		10 mg. fraction A	3–6	15	3.1	5.3
		10 mg. fraction A†	4	9	2.1	4.6
		3 mg. crystalline growth I	3	9	2.5	3.8
		3 mg. crystalline growth II	3–4	9	3.5	5.4
			24	8	2.8	5.2
Park and Daughaday[50]	hypophysectomized	none	3	108	4.9	6.7
		10 μg. fraction A	3	15	3.7	6.3
		1 μg. fraction A	3	5	4.3	6.5
		none	3	3	4.7	—
		10 μg. fraction A-1	3	3	3.5	—

* For details of experimental procedures, see the original publications.
† Rats also injected with Upjohn lipo-adrenal cortical extract, 0.25 ml. each.

one was transferred for one minute to an insulin solution and washed with fresh medium. Both were then incubated in Ringer-phosphate glucose. They found that the difference in final glycogen content between the control and the insulin-treated sample was smaller after the pituitary pre-treatment than the difference produced by the same insulin treatment in controls never exposed to pituitary extracts. The extent to which this smaller difference in final glycogen content may be attributable to an elevated glycogen deposition produced as a result of the insulin-like activity of pituitary extracts themselves (see below) cannot be determined from these experiments.

The observations of the Stadie group upon glycogen deposition following combined pituitary and insulin treatment are similar in two respects to ours upon glucose uptake. (a) Highly purified growth hormone cannot substitute for crude pituitary extracts *in vitro*, but glycogen formation

after the transient exposure to insulin is reduced in diaphragms from rats injected 24 hours previously with growth hormone. In our experiments, glucose uptake and the increase produced by a small concentration of insulin are reduced by injection of growth hormone 24 hours previously (see TABLE 8). (b) The pituitary effect upon either glycogen deposition or glucose uptake is small or absent with diaphragms from adrenalectomized rats[66] or from hypophysectomized-adrenalectomized rats,[49] suggesting that some adrenal factor is required.

Anti-insulin effects of crude pituitary extracts have been reported by Corkill and Nelson[14] and by Ottaway and Smith.[48]

Another experimental approach to the question of action of growth hormone upon glucose utilization has been especially studied by the Young group. They found[15] that repeated doses of about 4 mg. per day of each of a number of samples of highly purified growth hormone would induce glycosuria in the cat after about 5 days. This has been confirmed on cats with three times recrystallized growth hormone[51] and on dogs by Campbell and coworkers.[7] The latter investigators concluded that this effect was proportional to the growth hormone content of their extracts.

In summary, it appears that there is a pituitary inhibitor of peripheral glucose uptake which is closely related to growth hormone but may not be identical with it. Growth hormone appears to produce only the inhibition which develops some 18 hours or more after injection, while cruder extracts can produce an inhibition of glucose uptake within three hours after injection. The suggestion arises that the inhibitor may be formed from growth hormone in the body.

3. Insulin-Like (Hypoglycemic Effects) of Growth Fractions. There is another effect of growth hormone on glucose uptake which has been encountered with the isolated diaphragm. For both practical and theoretical reasons, it would be advantageous to add the pituitary fractions directly to the incubation media in which the diaphragm is suspended. When this was done by Park, Brown, Cornblath, and Daughaday,[51] it was found that certain samples of highly purified growth hormone, as well as of Fraction A, produced an *increase* in glucose uptake of diaphragms from hypophysectomized rats. The effect is not observed with diaphragms from normal rats under their test conditions (TABLE 10). With higher glucose concentrations, even normal diaphragms were found to respond. From this observation, it would be expected that such pituitary products should be hypoglycemic. Injection of 190 microgram doses into eviscerated hypophysectomized rats gave a fall in blood sugar and an increase in rate of glucose uptake of the excised diaphragm (TABLE 11). The stimulating effect on glucose uptake is marked at one hour after injection and tends to fall off thereafter.

It is obvious that this short-term insulin-like activity, when present in either the diaphragm test system or the cell-free hexokinase test system, may obscure the effect of the inhibitor substance, particularly the effort to demonstrate inhibitor effects shortly after introduction of the pituitary factor into the test system.

Accordingly, efforts are being made to define the properties of the insulin-like substance and to separate it from the inhibitor.

The hypoglycemic activity of various pituitary fractions has been assayed against insulin in the eviscerated rat and in the adrenalectomized mouse. One hundred micrograms of sample no. 10750 of four times recrystallized growth hormone, for example, is equivalent to a dose of insulin between 10^{-3} and 10^{-2} units, *i.e.*, to 0.05 to 0.5 micrograms insulin.

TABLE 10

In Vitro EFFECTS OF A SAMPLE OF FOUR TIMES RECRYSTALLIZED GROWTH HORMONE UPON GLUCOSE UPTAKE OF DIAPHRAGMS FROM NORMAL AND HYPOPHYSECTOMIZED RATS*

Additions to incubation medium	*Glucose uptake, mg. wet tissue per hr.*	
	diaphragms from normal rats	*diaphragms from hypophysectomized rats*
None	3.9 (12)†	4.7 (9)
100 µg. growth hormone per ml.	3.7 (6)	5.8 (5)
0.001 unit insulin per ml.	5.8 (6)	6.8 (4)

* From Park, Brown, Cornblath, and Daughaday.[51]
† The number of rats used is given in parentheses.

TABLE 11

EFFECT OF INTRAVENOUS INJECTION OF FOUR TIMES RECRYSTALLIZED GROWTH HORMONE INTO THE EVISCERATED HYPOPHYSECTOMIZED RAT ON THE BLOOD SUGAR OVER A 70-MINUTE PERIOD AND THE GLUCOSE UPTAKE OF THE DIAPHRAGMS REMOVED AT THE END OF THIS 70-MINUTE PERIOD*

Injection	*Fall in blood sugar*	*Glucose uptake by diaphragm, mg. per gm. wet tissue per hour*
	mg. %	
Medium only, control (4)†	22	4.7
190 µg. growth hormone (4)	92	6.4
0.01 Units insulin (free of hyperglycemic factor (3)	91	7.2
190 µg. crystalline bovine serum albumin (4)	18	5.0

* From Park, Brown, Cornblath, and Daughaday.[51]
† The number of rats used is given in parentheses.

This insulin-like activity may be related to the fall in blood sugar which is observed after administration of relatively large doses of growth hormone to normal or adrenalectomized rats[46] or to hypophysectomized dogs.[17] This fall in blood sugar does not require presence of the pancreas, as it is observed in diabetic dogs.[17] In whole animals, the hypoglycemic effect wore off in a few hours and was succeeded by development of relative insulin resistance.

B. CARBON DIOXIDE PRODUCTION, RESPIRATORY QUOTIENT. The respiratory quotient of the glucose-fed rat can be reduced from 0.95 to 0.7 by injection of anterior pituitary fractions just prior to the glucose feeding. Greaves,

Freiberg, and Johns[27] prepared fractions which were active at a dose of about 80γ per 100 gm. rat.

This problem has been taken up again by Dr. Lillian Recant, who has made a number of new observations.[54] When injected into the whole animal, growth hormone produced a lowering of R.Q. at doses comparable with those required for samples of Q fraction prepared by slight modifications of the Greaves method (TABLE 12).

TABLE 12

AMOUNTS OF VARIOUS PITUITARY FRACTIONS REQUIRED TO REDUCE THE R.Q. OF THE GLUCOSE-FED NORMAL RAT FROM 0.97 TO 0.80*

Fraction injected intraperitoneally 1 hour before feeding rat 1 gm. glucose/100 gm.	*Minimum dose of protein required to reduce R.Q. to 0.80*
	mg. per 100 gm. rat
Fraction A	46
Fraction A-1	10
Growth hormone, four times recrystallized	0.030
Greaves Q factor	0.013
ACTH (Armour)	ineffective at 3 mg.

* From Recant.[54]

TABLE 13

In Vitro R.Q. AND GLUCOSE UPTAKE OF RAT DIAPHRAGMS FROM RATS INJECTED INTRAPERITONEALLY ONE HOUR BEFORE DIAPHRAGM REMOVAL*

Injection	*Substrate, in vitro*	*Average R.Q.*	*Glucose uptake, mg. wet tissue per hour*
None	none	0.70	—
	glucose, 350 mg.%	0.83	5.84
	glucose, 350 mg.% + 1 unit insulin/ml.	—	8.39
500 μg. Q fraction	glucose, 350 mg.%	—	6.23
	glucose, 350 mg.% + 1 unit insulin/ml.	—	7.55
600 μg. Q fraction	glucose, 350 mg.%	0.71	—
	glucose, 350 mg.% + 1 unit insulin/ml.	0.68	—

* The rats were fasted 24 hours prior to the injection. The Q fraction was prepared according to Greaves[27] (from Recant[54]).

The isolated diaphragm also had a lowered R.Q. when removed from a rat injected one hour previously with Q fraction or growth hormone (TABLE 13). Moreover, the lowering of R.Q. was produced by addition of the growth hormone to the diaphragm *in vitro*. This R.Q.-depressing effect of the pituitary fractions was not reversed by addition of insulin to the medium in which the diaphragm was suspended (TABLE 13). Under the same circumstances, insulin still produced a significant increase in glucose uptake. This indicates that the locus of action of the pituitary fractions in depressing R.Q. may be different from, and subsequent to, the hexokinase reaction.

These observations on the R.Q.-depressing factor of the pituitary have

to some degree paralleled new studies upon the pituitary myoglycostatic factor by Russell and her collaborators. It was shown, some years ago, that the fasting hypophysectomized rat loses muscle glycogen more rapidly than the fasting normal.[57] This abnormal loss of muscle glycogen can be prevented entirely by administration of anterior pituitary extract,[59] and the R.Q. is depressed to normal at the same time.[20] Further, the myoglycostatic effect of crude pituitary extracts could be demonstrated by enhanced deposition of muscle glycogen in fed normal rats.[58] Illingworth and Russell[33] have now found that a single intraperitoneal injection of 1 or 2 mg. crystalline growth hormone per 100 gm. into fasted normal adult rats produced an increased accumulation of glycogen in gastrocnemius, heart, and diaphragm muscles at the end of six hours.

The effects of present samples of crystalline growth hormone upon glucose utilization in muscle appear, therefore, to fall under the following headings: (a) An action to favor glycogen accumulation. This effect becomes apparent soon after injection and is made up of increased glucose uptake (an insulin-like effect) and decreased glycogen breakdown to CO_2 (myoglycostatic effect and depression of R.Q.). (b) An action in the direction of decreased glucose uptake and decreased response of muscle to insulin. This effect becomes apparent only when several hours are allowed to elapse after growth hormone injection. The time factor raises the question whether the inhibitor of glucose uptake may be due to a product formed in the body from the entity now regarded as growth hormone.

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THE COMBINATION OF INSULIN WITH TISSUE*

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For some years past, the research activities of the Department of Research Medicine have centered around the question of the chemical action of insulin. This subject has excited the interest of many workers for more than seventy-five years and at present is being actively pursued in many laboratories in a great variety of experimental ways. Recently, we have uncovered a new phenomenon, which we have termed "the combination of insulin with tissue." We have performed many experiments to determine how significant this phenomenon is in the problem of the chemical action of insulin, and it is this work that I propose to discuss in this paper.

The genesis of our work resulted from the emphasis which was given to homogeneous systems of enzymes in the study of the problems of intermediary metabolism. Systems of tissue extracts and substrates in simple aqueous solution were used. Great progress became possible by the successful isolation from such homogeneous systems of individual enzymes, many in crystalline form, and the demonstration of the specific chemical action which they, together with their coenzymes, catalyzed. By virtue of the great success of these methods, it became more or less axiomatic that complete understanding of metabolic pathways could not be achieved unless they were demonstrated in model or synthetic systems composed of highly purified enzymes. It was natural, therefore, to adopt the concept that the effect of hormones upon enzymatic systems could best be demonstrated in similar homogeneous systems which were free of intercellular boundaries or of cellular structures.

Studies of the effect of hormones, particularly insulin, upon such homogeneous enzyme systems from normal and diabetic animals were reported in the literature, but an evaluation of this evidence, together with our own experiments, convinced us that there was no unequivocal evidence that insulin had any effect upon any enzyme system when it existed in cell-free or homogeneous systems.

This brought us to the point where we were forced to reconsider the nature of the problem of insulin action. Accordingly, we formulated a new working hypothesis as the basis for further experimentation. This hypothesis consisted of two parts: (1) retention of cellular integrity is necessary for hormonal action upon enzyme systems (that is to say, the system, instead of being homogeneous, is heterogeneous); (2) the hormone itself must engage in the heterogeneity (this is another way of saying that the hormone combines with some receptor of cellular structure). Certain *a priori* considerations made this hypothesis attractive: (1) the necessity of postulating a trimolecular reaction, a rare occurrence, among enzyme, hormone, and substrate would be eliminated; and (2) a combination of

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enzyme and hormone generating a new entity would help explain changes of specificity and efficiency of catalysis.

At all events, the combination of insulin with tissues proved to be easy of demonstration. For this purpose, we used the isolated rat diaphragm, which responds to insulin in a characteristic way by synthesizing more glycogen when equilibrated with glucose. In our early experiments, we merely dipped the diaphragm, freshly removed from the rat, in an insulin solution. The diaphragm was then thoroughly washed in saline and finally equilibrated in a glucose solution for about one hour. Invariably, we found that the hemidiaphragm which had been exposed to insulin for a brief period synthesized more glycogen than did its paired control hemidiaphragm which had not received treatment with insulin.

We interpreted this finding to mean that, during the brief preliminary exposure of the diaphragm to insulin, there occurred a chemical combination between the insulin and receptor groups on the diaphragm. The combined insulin resisted the dissociating action of prolonged washing with saline. This combined insulin exerted its characteristic effects in changing the

TABLE 1

STANDARD TECHNIQUE FOR MEASURING COMBINATION OF INSULIN WITH RAT DIAPHRAGM*

I—Fixation period 1 minute; insulin 0.1 unit/ml.	*II—Washing period* Twice with 25 ml. of medium.
III—Assay period 90 minutes; with 0.2% glucose	*IV—Analysis* Measure of insulin combined is "insulin effect" = extra glycogen synthesized by test hemidiaphragm.

* Test and control hemidiaphragms treated alike except for insulin in fixation period.

metabolic pattern of the diaphragm so that, when it was subsequently equilibrated with glucose, it responded by synthesizing extra glycogen.

The technique became formulated in the more or less constant pattern shown in TABLE 1. In a first, or fixation, period, the diaphragm was agitated for a period varying from ten seconds up to five minutes in a solution containing insulin. The diaphragm was then allowed to drain for ten to fifteen seconds, the hanging drop was removed by filter paper, and the diaphragm was washed in 25 cc. of saline for a period of one minute. This washing was repeated once. The diaphragm was transferred to a Warburg or similar vessel containing glucose for a final assay period, usually 90 minutes long. The diaphragm was then analyzed for glycogen. The results are expressed as the "insulin effect," or the amount of extra glycogen synthesized over that by the paired hemidiaphragm, which has been carried through similar steps with the exception of the exposure to insulin.

There is no possibility that insulin carried over physically could be the cause of this effect. The dilutions achieved by the customary washing dilute the insulin far beyond any conceivable concentration to give an effect. We have, in addition, equilibrated the control hemidiaphragm, suitably identified by a punch mark in the same vessel with the insulinized hemidiaphragm, with results that are identical with those by our customary

procedure. We have washed insulin-treated diaphragms for as long as one hour. The combined insulin withstood a prolonged washing without dissociation as shown by significant insulin effects.

FIGURE 1 shows an experiment of similar character. Each one of the points represents the mean insulin effects obtained from five or six pairs of rat hemidiaphragms. In this experiment, the period of equilibration in the glucose after the preliminary exposures of insulin varied from fifteen minutes up to one hundred and twenty minutes. Up to sixty minutes,

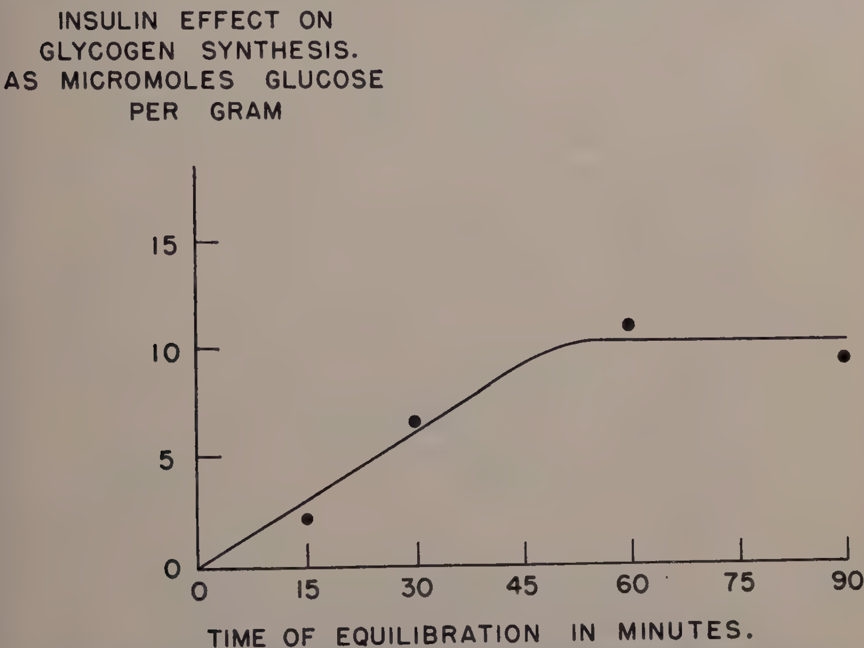


FIGURE 1. Extra glycogen synthesis (insulin effect) by insulinized hemidiaphragm equilibrated for varying times in glucose medium. Each point represents the mean insulin effect obtained from determinations on 5–6 hemidiaphragms exposed for one minute to insulin at 0.1 unit per ml. The paired untreated hemidiaphragm served as control. Reprinted from STADIE, W. C., N. HAUGAARD, A. G. HILLS, & J. B. MARSH. 1949. Am. J. Med. Sci. **218:** 265.

the effect of the combined insulin in causing extra glycogen synthesis falls on a straight line. This means that, during this 60 minutes, the bound insulin did not dissociate from the diaphragm. Otherwise, the extra glycogen synthesis would have decreased. The point at ninety minutes is off the line, indicating, perhaps, that insulin has dissociated or that the ability of the diaphragm to respond to insulin has fallen off.

Following is a summary of some of the chemical aspects of our experiments. We have demonstrated the binding of insulin to rat hemidiaphragm using a variety of insulin preparations. These are amorphous insulin, zinc-free insulin, crystalized insulin, and protamin zinc insulin. There is no apparent effect of pH upon the binding over a range of pH 6–8. The question of the effect of more acid pH upon this phenomenon will be discussed in

more detail later. Increasing temperature appears to increase the binding of insulin. These experiments were done by varying the temperature during the fixation period, but the temperature during the assay period was always 38°. This observation is in favor of the concept that the binding is chemical in character, since it has such a high temperature coefficient. The combination of insulin with the diaphragm may be formulated by physicochemical concepts. This is indicated by the material in FIGURE 2.

As a first approximation, assume that there are receptor areas located on structural units of the cell which are capable of combining with insulin. Some of these will be combined and others free. Assuming that the com-

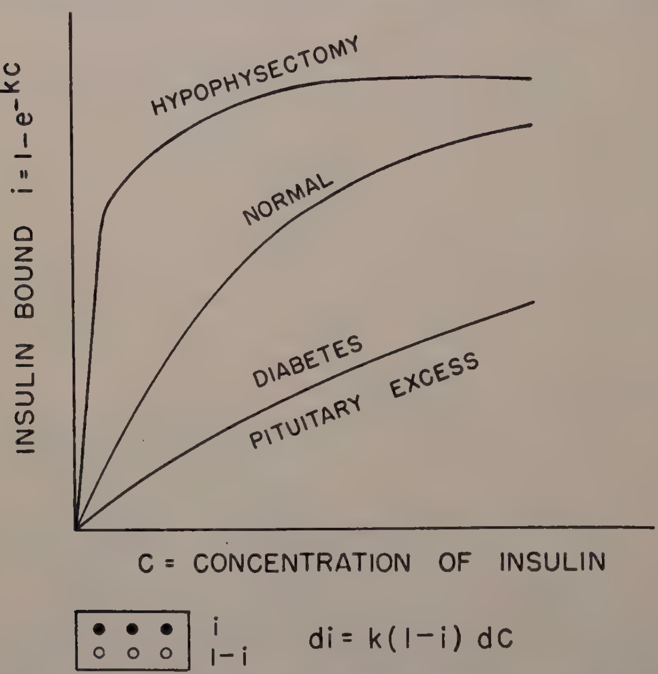
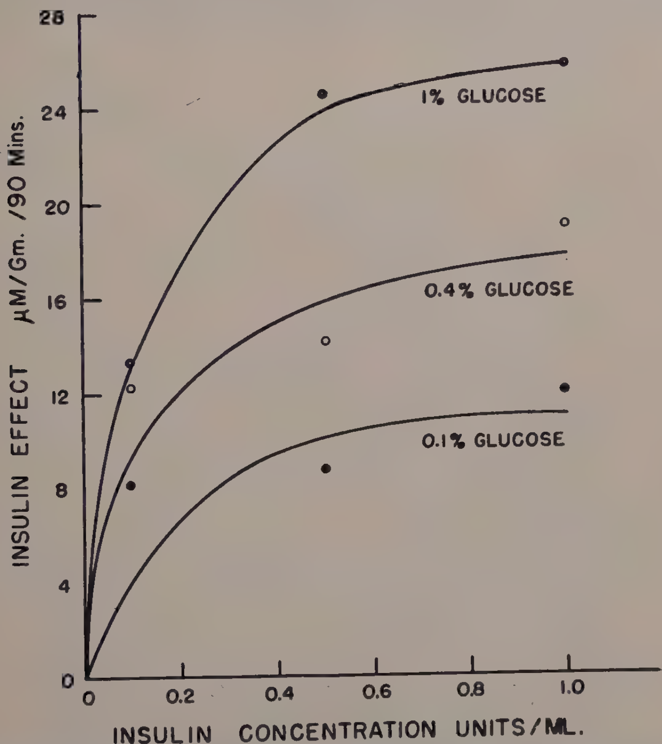


FIGURE 2. Schematized curves of "insulin bound" (assumed proportional insulin effect) in various endocrine states.

bination of insulin is proportional to the free areas, 1-i, and the insulin concentration, we obtain the differential equation expressed on the right of the slide. Upon integration, we obtain the schematized curves shown in the figure, which represents the "combined insulin" as a function of insulin concentration. The curves are characterized by (1) a more or less rapid increase of bound insulin with increasing concentration, and (2) a maximum value at high concentrations.

These curves resemble those obtained experimentally, if we assume that the insulin effect on glycogen synthesis is proportional to "bound insulin." The endocrine state shifts the curves: to the right, in the case of diabetes and excess pituitary due to injection of APE; and to the left, in the case of the hypophysectomized rat. These changes could be reflections of altera-

tions in the mass action constant, k, of the equation. We cannot push this analysis too far at present. We are entirely dependent upon an indirect method to measure "combined insulin," namely, the measurement of extra glycogen synthesis. Hence, we cannot state whether these endocrine disturbances have altered the ability of the diaphragm to combine with insulin or whether the combined insulin is unaltered in amount but has had its action enhanced or diminished by endocrine imbalances. Isotopically la-



FIGURE, 3. The effect of combined insulin on glycogen synthesis as a function of the insulin concentration during the fixation period. Three different concentrations of glucose were used during the assay period (5 minutes). Each point represents the mean insulin effect obtained from determinations on 5–6 pairs of hemidiaphragms. Reprinted from STADIE, W. C., N. HAUGAARD, & J. B. MARSH. 1951. J. Biol. Chem. **189:** 53.

belled insulin would be of great help in studying these physico-chemical aspects, and we have started experiments of this type.

We have studied this phenomenon of binding in a great variety of ways. We have varied the insulin concentration during the preliminary period, the time of the fixation period, and the concentration of the glucose during the assay period. The limitations of space allow the inclusion of only a few of our experiments which illustrate the type of studies done. In FIGURE 3 is shown the insulin effect as the function of the concentration during the fixation period. By assuming that this insulin effect measures the amount of combined insulin, we get characteristic curves. In the lower curve, when the glucose concentration is low it becomes the limiting factor, and

the insulin effect levels off at a low value. The contrary holds true when the glucose concentration is high. In this case, the high concentration of glucose tends to make the insulin the limiting factor, so that quite high values of insulin effect are obtained as the insulin concentration increases.

In FIGURE 4, as before, the time of exposure to insulin in the fixation period is the same, namely, five minutes. The three concentrations of insulin were .1, .5, and 1.0 unit per ml. In the lower curve, it is evident that the amount of insulin that is bound is relatively small, and it becomes

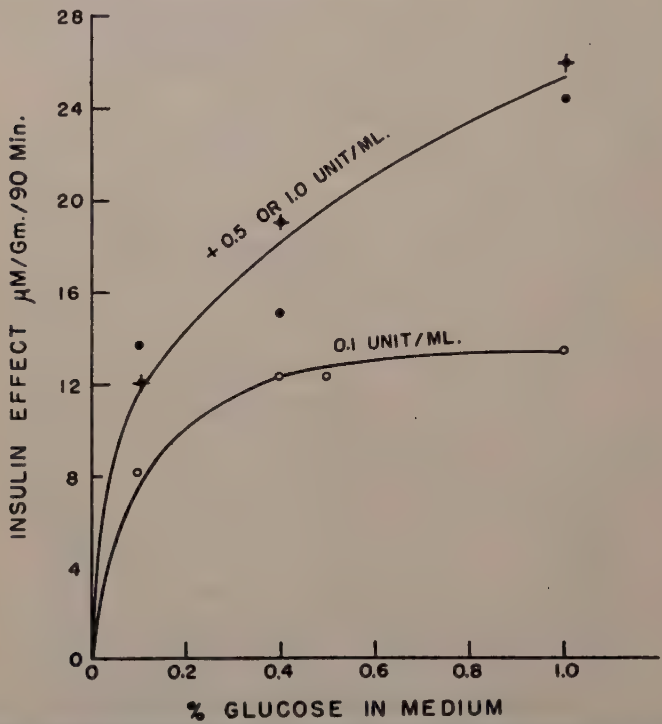


FIGURE 4. The effect of combined insulin on glycogen synthesis as a function of the glucose concentration during the assay period (5 minutes). Each point represents the mean insulin effect obtained from determinations on 5–6 pairs of hemidiaphragms. Reprinted from STADIE, W. C., N. HAUGAARD, & J. B. MARSH. 1951. J. Biol. Chem. 189: 53.

the limiting factor, so the insulin effect levels off despite the great increase in glucose concentration in the assay period. In the upper curve, glucose becomes the limiting factor, the insulin bound being approximately the same whether the concentration is ½ or one unit per m. In consequence of this, the insulin effect continues to increase as the glucose in the medium is increased.

In FIGURE 5 is shown the rapidity of the binding of insulin to tissues. The exposure during the fixation period is twelve seconds, at varying concentrations of insulin. The assay was done in three different concentrations of glucose. Significant effects are obtained at low concentrations of insulin and glucose. This figure indicates the very interesting phenomenon of a rapid binding of insulin to receptor groups on the diaphragm.

This section of our experiments can be summed up by saying: (1) For the first time, a chemical reaction finite in time involving a hormone has been demonstrated. (2) The chemical combination of an endocrine substance with tissue has been shown to occur. (3) This combination can be formulated in physico-chemical terms on assumptions involving mass action concepts.

We have previously shown that variation of pH over a relatively narrow range around neutrality (4–6) had no apparent effect upon the binding of

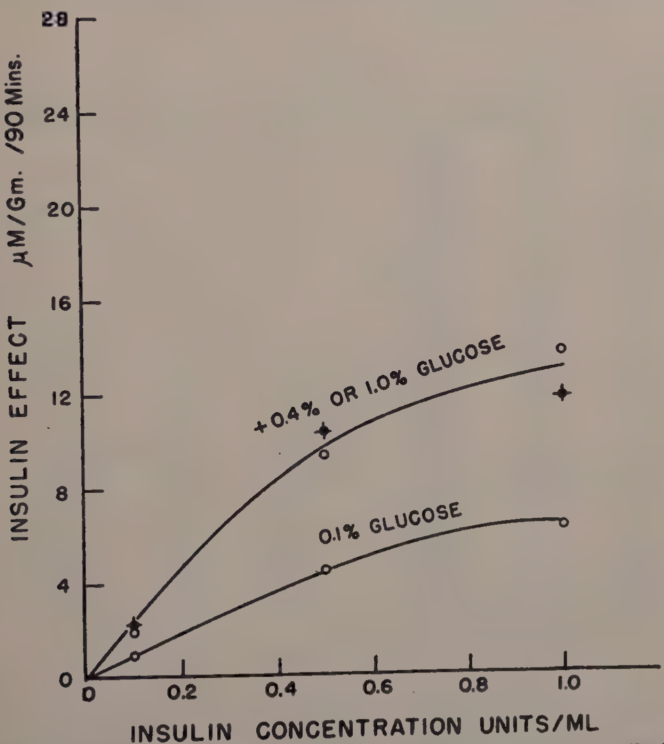


FIGURE 5. The effect of combined insulin on the glycogen synthesis by rat diaphragm following exposure to insulin for 12 seconds. Each point represents the mean insulin effect obtained from determinations on 5–6 pairs of hemidiaphragms. Reprinted from STADIE, W. C., N. HAUGAARD, & J. B. MARSH. 1951. J. Biol. Chem. **189:** 53.

insulin. We were more interested, from a theoretical point of view, in studying the binding of insulin when the insulin solution was at pH 2.0. It has been well established by Oncley and others that at pH 2.0 insulin exists solely as a monomer of molecular weight 12,000. We were interested in testing whether insulin in this form would combine with diaphragm. Accordingly, we equilibrated diaphragms for 30 seconds with insulin (0.1 μ per ml.) in saline medium containing 0.02 N HCl to give a pH of 2.0. Control hemidiaphragms were similarly treated but without insulin.

Following the exposure to insulin, customary washings were done, and the diaphragms were equilibrated in glucose solution at pH 6.8. The results are quite consistent (TABLE 2). There is an extra synthesis of gly-

cogen in the hemidiaphragms which had been exposed to insulin. This means that the monomer combines with the diaphragms and exerts its customary metabolic effects. The significance of this experiment is uncertain. It is to be noted, however, that the insulin effect is the same regardless of whether the pH during the fixation period is 2 or 7. This might be taken to mean that it is the monomer which is bound throughout this pH range and that it, rather than the trimer, is the biologically active form. Since the insulin solutions used are very dilute, the existence of the monomer through this pH range would be consistent with physicochemical evidence.

TABLE 2

COMBINATION OF MONOMERIC INSULIN (MW 12,000) WITH NORMAL RAT DIAPHRAGM*

No.	*Final glycogen μM/gm.*		
	Control	*Insulin treated*	*Insulin effect*
1	22.6	28.5	6.1
2	20.8	24.4	3.6
3	22.7	33.3	10.6
4	20.2	29.0	8.8
5	23.3	31.7	8.4

Mean SEM 7.5 1.21
$P \ll 0.001$

* Fixation period—30 seconds; medium—NaCl in 0.01M HCl (pH = 2.0); washed twice in saline; assay period—90 minutes, 38°C, 0.4% glucose.

TABLE 3

THE EFFECT OF COMBINED INSULIN IN VARIOUS ENDOCRINE STATES

1—Diminished in Alloxan—diabetic rats.
2—Increased in hypophysectomized rats.
3—Unchanged in adrenalectomized rats.
4—Diminished by prior injection of pure growth hormone but only in presence of adrenals.
5—Diminished by pretreatment *in vitro* with pituitary factor *not* growth hormone or ACTH.

TABLE 3 shows that departures from the normal state of endocrine balance produce marked changes in the behavior of the isolated diaphragm when tested by the technique outlined. For example, in the rat made severely diabetic as judged by level of blood sugar and glucose output, the response of the insulin-treated hemidiaphragm vanishes completely. Similarly, if normal rats are injected intraperitoneally with a crude saline extract of the anterior pituitary some 12 to 18 hours before the experiment, they yield diaphragms which fail to respond to insulin. There are two possible interpretations of these results. We can say that changes in the diabetic muscle or following APE injection have produced alteration in the receptor mechanism of the muscle, diminishing its ability to combine with insulin. Hence, the insulin effect, when measured in the presence of

glucose, decreases or vanishes. Alternatively, we can say that the insulin has combined as before, but that the diabetic state or the APE have altered the ability of the diaphragm to respond. We are currently using isotopically labelled insulin which we hope will yield evidence to decide between these alternatives, but these experiments are too preliminary for discussion.

Further studies on the relation of the pituitary to the phenomenon of binding are shown in FIGURE 6. This gives data on hemidiaphragms from normal and hypophysectomized rats. As indicated in the figure, the rats

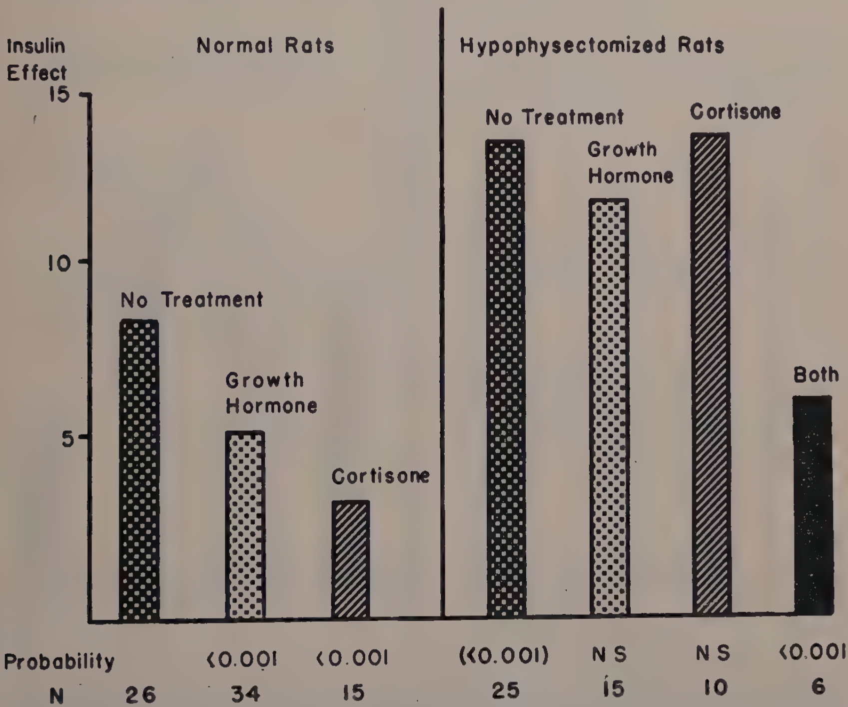


FIGURE 6. Comparison of the effect of combined insulin in normal and hypophysectomized rats. N = the number of determinations of the insulin effect on paired hemidiaphragms. P = probability that the difference from "no treatment," (or "normal: no treatment" in the case of the "no treatment: hypophysectomized") is due to chance.

were either untreated or injected 12 to 18 hours before sacrifice with growth hormone or cortisone. The assay for the insulin effect was done in glucose at 0.4 per cent for ninety minutes. N. indicates the number of paired hemidiaphragms in each series, and the probability indicates whether the difference in the insulin effect is due to chance. In the normal rat, the prior injection of growth hormones or cortisone 12 to 18 hours before the experiment produces a very significant decrease of the insulin effect. In the case of the hypophysectomized rat, there is a markedly increased response of the diaphragm to insulin, demonstrating *in vitro* the greatly increased sensitivity of the hypophysectomized animal for this hormone. In contrast to the normal rat, the injection of growth hormone or cortisone separately produces no change in the response of the diaphragm. When both

are injected together, however, there is a significant decrease of the response of the diaphragm to insulin. This points to a synergism between these two hormones, for which there is other evidence in the literature.

Similar experiments were done upon adrenalectomized rats (FIGURE 7). Adrenalectomy itself had no significant effect upon the response of the diaphragm to insulin. Neither growth hormones nor cortisone separately had any effect. When growth hormone and cortisone were injected to-

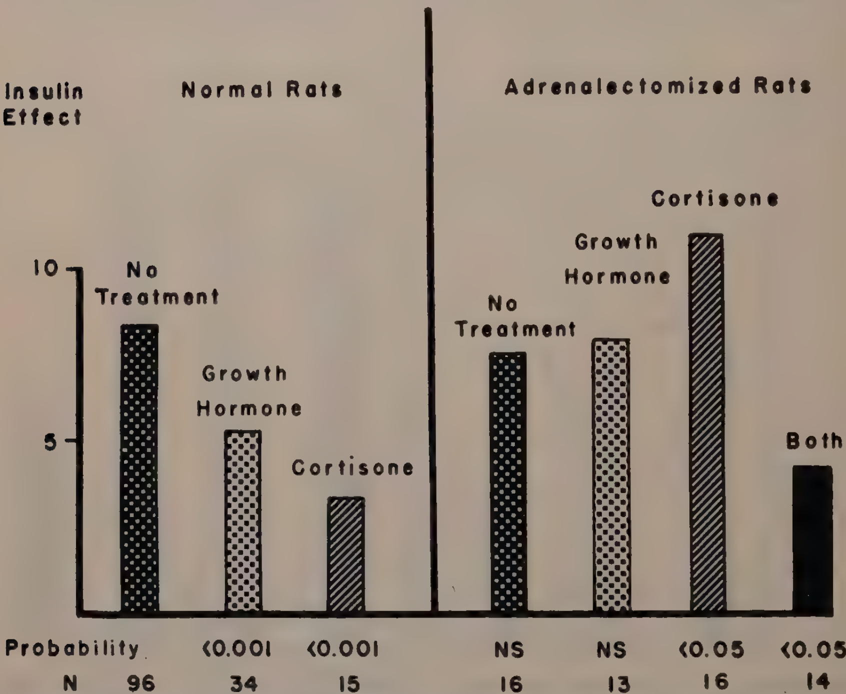


FIGURE 7. Comparison of the effect of combined insulin in normal and adrenalectomized rats. N = number of determinations of the insulin effect on paired hemidiaphragms. P = probability that the difference from "no treatment," (or "normal: no treatment" in the case of "no treatment: adrenalectomized") is due to chance.

gether, however, there developed a decrease in the insulin response of the diaphragm.

The possibility that competitive inhibition by some pituitary factor is the cause of the diminished response of these diaphragms to insulin was investigated by a series of experiments included in TABLE 4. These experiments differed from the previous ones in this respect: each hemidiaphragm was first pretreated with a crude saline extract of anterior pituitary for a brief period of time. The hemidiaphragm was then washed thoroughly in saline and, together with an untreated paired control, was subjected to insulin treatment and assayed for insulin effect according to our standard method. We see, from the data in the table, that as little as one minute pretreatment with the crude anterior pituitary is sufficient to prevent a subsequent response to insulin. In contrast, we found that a purified

preparation of growth hormone, a variety of tissue extracts such as liver and muscle, and various serum protein fractions, are without effect when tested in the same way as these APE extracts. This experiment would indicate that there is some factor in the pituitary, which is not growth hormone, which itself combines with the diaphragm or so alters it that it is subsequently unable to respond to insulin when tested by our standard technique.

It was important to determine how general it was—this phenomenon of combination of insulin with tissue. We therefore extended our work to

TABLE 4
INHIBITION OF COMBINED INSULIN BY PRETREATMENT OF DIAPHRAGM WITH APE*

	N	*Time of pretreatment (min.)*	*Mean insulin effect μM/gm.*
Normal controls	45		5.8
APE treated	6	1	2.9†
	14	5	1.0†
	6	5	1.4†

* Pure growth hormone, liver extract, muscle extract, and several serum protein fractions have no significant effect when used to pretreat diaphragms.
† Significantly less than controls.

TABLE 5
CHEMICAL COMBINATION OF INSULIN WITH RAT ADIPOSE TISSUE*

Oxygen uptake μM/gm./hr.	*% Increase with insulin*
4.7	19
4.9	35
6.0	2
9.2	9
15.2	13
9.4	17
7.1	17
7.9	29
7.5	11
Mean: 7.9 ± 1.06	17 ± 3.4†

* Five minutes pre-equilibration with 1 unit insulin per ml. at 38°C. Two washings in 25 ml. medium Oxygen uptake determined in the presence of glucose.
† Significantly different from zero ($P < 0.001$).

include tissue other than muscle. There are relatively few tissues which can be made to demonstrate an insulin effect by *in vitro* methods. One of these is rat adipose tissue (TABLE 5). Contrary to popular notion, adipose tissue is quite active metabolically. Under certain circumstances, it has a high RQ, indicating, perhaps, that there is a synthesis of fat from acetate or glucose. In addition, it responds to insulin with an increased uptake of oxygen. Drs. Haugaard and Marsh in our laboratory have shown that when this adipose tissue is pretreated with insulin, using the technique already described, it shows an increased metabolism, indicating that it, similar to the diaphragm, has combined with insulin, and that the combined insulin exerts a characteristic effect upon the metabolism.

We investigated another tissue for its ability to combine chemically with insulin. This is the lactating mammary gland of the rat. This tissue is known to respond to insulin *in vitro* by increasing its respiratory quotient when acetate is present as a substrate. This is taken to mean that the synthesis of fat is accelerated by insulin. Dr. Hills, using a technique similar to that used for the diaphragm, has studied the possibility of the combination of insulin with this tissue. The results are shown in TABLE 6. We see from the data in this table that pre-exposure for five minutes and two minutes gives a significant effect upon the RQ. In the case of one minute exposure, the effect is marginal. In other words, it is evident, as in the case of the muscle and adipose tissue, that insulin has combined and in its combined form has exerted its customary effect.

In closing, I would like to summarize very briefly. It is our opinion that there is no satisfactory evidence to indicate that hormones have any effect upon enzymes in homogeneous systems. Our working hypothesis is that cellular structure is necessary for the effect of insulin and perhaps other hormones. Our experiments show that insulin combines with tissue in a manner formulated by physico-chemical concepts based on mass action. The precise mechanism of binding remains to be determined. Apparently, the combination of insulin with tissues is a general phenomenon, since tissues of widely different character (*viz:* muscle, adipose, and mammary tissue) are capable of combination. The types of metabolic processes affected also vary widely. Whether combination is a prerequisite for the action of insulin remains to be determined, but either the combination of insulin or the action of the bound insulin is markedly affected by the diabetic state and by pituitary and adrenal factors. In addition, we have evidence that a pituitary factor, presumably not growth hormone or ACTH, combines with the isolated rat diaphragm and subsequently nullifies the usual effect of insulin.

Table 6

The Effect of Combined Insulin upon the Respiratory Quotient of Slices of Lactating Mammary Gland from Normal Rats

No. of rats	*No. of determinations*	*Time of pre-equilibration minutes (ins. = 1 unit per ml.)*	*Control mean RQ*	*Insulin effect of RQ mean ± sem*	*t*
7	32	5	1.58	+0.30 ± .034	8.61
1	6	2	1.31	+0.20 ± .059	3.40
2	13	1	1.37	+0.12 ± .026	4.66

We feel that insulin combination is a central factor in the problem of insulin action and is an interesting field for further study.

Acknowledgment. The experimental work reported here was done in collaboration with Dr. Nils Haugaard, Dr. Julian Marsh, and Dr. A. Gorman Hills.

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THE DISPOSITION OF GLUCOSE BY THE EXTRAHEPATIC TISSUES*

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The use of radioactive carbon as a tracer incorporated in glucose offered a device whereby the usual uncertainties accompanying studies of the disposition of glucose in the eviscerated organism could be reduced or eliminated. Uniformly labeled C14 glucose was prepared in a good yield and with a high activity by biosynthesis using a procedure described elsewhere.[1]

Rabbits were used and eviscerated as previously described.[2] With the aid of frequent blood sugar determinations, the blood sugar was maintained at a normal level for 8 or more hours by the constant injection of C14-labeled glucose. The glucose solution used for the constant injection was calculated and prepared to have the same specific activity as that of the circulating plasma glucose resulting from the injection of a priming dose of C14-labeled glucose. The purpose of the priming dose at the start of the experiment was to produce a rapid equilibrium between the specific activity of the body glucose and the glucose to be used for the constant injection. For this calculation, the space of the body glucose is required, and we found it to be equivalent to 25 per cent of the body weight.[3] The technical and analytical methods used have been detailed elsewhere.[3–5]

Glucose Utilization by the Extrahepatic Tissues. In the past, the preferred method for measuring the amount of glucose utilized by the extrahepatic tissues has been that of determining the rate at which glucose must be injected in order to keep the blood sugar at a normal constant level. The evisceration eliminates the actions of the liver, pancreas, and gastrointestinal tract—all variables which would be difficult to control. This method assumes that the liver is the sole source for the new formation of glucose, a fact which, we now know, is not true. The kidney may form glucose[6] in the absence of the liver, but, since it does so only if there is a hypoglycemia, this source may be ignored in these experiments.

It should be stressed that the old "utilization" rate was really the disappearance rate of glucose, and it did not tell us anything about the relative magnitude of the different routes of disposal of this glucose by the body, *viz.*, oxidation to CO_2 and H_2O, partial oxidation and conversion to intermediate compounds, and conversion to storage substances like glycogen, protein, and fat. Under partial oxidation substances, we must distinguish between those substances that are on the way to complete oxidation, and those that might be called "dead-end" glucose derivatives, which, in the normal animal, are reconverted to glucose by the liver. Using C14 glucose enables one to distinguish between glucose which disappears and that which is oxidized. Typical results comprise TABLE 1. Not more than a fifth of the glucose which disappears in the eviscerated organism is actually

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oxidized. A comparison of the specific activity of the expired CO_2-(342) and that of the injected glucose-(3050) shows that hardly more than 11 per cent of the expired CO_2 comes from the combustion of the circulating glucose over an eight-hour period. Even at the end of this time, the expired CO_2 includes less than a third of the glucose which is disappearing.

It is apparent that a large amount of the glucose which disappears goes into routes other than immediate oxidation. TABLE 2 lists the disposal of the glucose which does not undergo combustion. The amount stored as fat and glycogen is quite small. Protein claims a significant amount. Considerable sugar went into the water soluble fraction of the carcass with lesser amounts in the urine that is not glucose, urea, or carbonate. Some of the latter may represent dead-end products of glucose that do not get to CO_2 in the absence of the liver. The water-soluble fraction may represent a form of storage or a metabolic pool in which the glucose was in transit to final oxidation. The results make it necessary to revise all calculations of tissue glucose "utilization" based on glucose disappearance in hepatectomized or eviscerated animals.

TABLE 1

COMPARISON OF GLUCOSE "DISAPPEARANCE" AND OXIDATION RATES IN EVISCERATED RABBITS

Animal	*Mg. glucose/kg./hour*	
	disappearance	*oxidized*
1	84	18
2	88	27
3	112	30
4	124	30

TABLE 2

DISTRIBUTION OF C14 IN THE EVISCERATED RABBIT AFTER A PERIOD OF EIGHT HOURS CONSTANT INJECTION OF C14-LABELED GLUCOSE (AVERAGE THREE ANIMALS)

	% of injected C14
Expired CO_2	17.2
Body CO_2	3.9
Circulating glucose	11.8
Water-soluble nonferm	31.2
Glycogen	2.6
Crude protein	6.1
Crude fat	1.3
Urine	4.1
Heart, lungs, kidneys	5.0
Total recovery	83.2

Effect of Blood Sugar Level on Rate of Glucose Oxidation. Before considering the influence of insulin upon the extrahepatic disposition of glucose, experiments were carried out on the effect of the blood sugar level. The widely held view promoted by Soskin[7] and by Bouckaert[8] that the concentration of glucose in the blood determines very largely the rate of utili-

zation of it makes any hormone regulation relatively unnecessary. According to them, the adjustment of glucose utilization to supply is largely automatically taken care of by a simple application of the law of mass action.

The results of our study of the effect of the blood sugar level upon the extrahepatic disposition of glucose are summarized in TABLE 3. There is, apparently, a slight increase in the rate of disappearance of glucose at the high blood sugar level and even less of an increase in the rate of glucose oxidation. They do not approach those obtained after the administration of insulin and are small enough to permit the conclusion that there is no

TABLE 3

EFFECT OF BLOOD SUGAR LEVEL ON RATE OF GLUCOSE OXIDATION IN EVISCERATED RABBITS. VALUES EXPRESSED AS PER CENT OF EXPIRED CO_2 FROM INJECTED GLUCOSE

Hr.	*Normal blood sugar level*			*High blood sugar level*			
	R-1	*R-2*	*R-3*	*R-4*	*R-5*	*R-6*	*R-7*
1	4	6	7	5	6	7	9
2	7	9	9	5	12	12	11
3	10	13	12	11	20	12	15
4	11	13	16	16	24	14	16
5	13	16	20	19	26	18	20
6	15	18	22	13	28	17	23
7	19	22	25		32	20	24
8	16		27		33	18	24
a*	241	199	166	212	258	224	207
b	58	62	63	118	77	61	78
c	145	200	197	340	540	600	510
d	156	190	204	326	590	600	495
e	1.98	1.80	1.86	1.97	2.16	1.76	2.38

* (a) Av. mg. glucose injected /kg./hg. (glucose disappearance rate).
(b) Av. mg. glucose oxidized /kg./hr.—5th to terminal hour inclusive.
(c) Starting plasma sugar—mg. per cent.
(d) Final plasma sugar—mg. per cent.
(e) Animal wt. in kilos.

general law of blood sugar level and oxidation rate of the sort which has been promoted.[7, 8]

Effect of Insulin on the Rate of Glucose Oxidation. An action which has long been ascribed to insulin is that of increasing the immediate oxidation of glucose. The chief argument used to support this claim has been that there is an increase in the respiratory quotient after the administration of this hormone. A rise in the respiratory quotient could occur as a result of an increased conversion of glucose to fat in some special organ or tissue while the rest of the tissues maintained a constant type of metabolism. The conversion of glucose to fat is ordinarily thought to take place mainly in the liver, although there is no certain proof of this. There is no experimental evidence that insulin will increase the combustion of glucose by the extrahepatic tissues. In order to determine whether the combustion of glucose in the liverless animal is increased by insulin, glucose labeled with C 14 was administered and its metabolic course followed in the usual manner.

Ten units of insulin were given intravenously each hour to the insulinized animals.

The data presented in TABLE 4 show conclusively that insulin increases not only the rate of glucose disappearance (glucose injected) but also the rate of glucose oxidation. Our results show that insulin acts on the extrahepatic tissues to increase greatly the combustion of glucose to carbon dioxide. This action is not immediate, there being a lag in the relative amount of C14 appearing in the expired carbon dioxide under the influence of insulin in comparison with the control animals. For the first time, we have positive proof that insulin increases the combustion of glucose by the extrahepatic tissues, a notion which has been popular since the early use of insulin, but which has never before had a sound experimental basis.

TABLE 4

COMPARISON OF GLUCOSE DISAPPEARANCE AND OXIDATION RATE IN EVISCERATED RABBITS WITH AND WITHOUT INSULIN ADMINISTRATION

Hrs.	*Glucose oxidized to CO_2 mg. per kg. per hr.*							
	control			*insulin*				
	R-1	*R-2*	*R-3*	*R-4*	*R-5*	*R-6*	*R-7*	*R-8*
1	15	22	13	10	3	53	38	15
2	17	29	29	10	15	92	95	38
3	21	29	47	11	38	153	177	67
4	22	31	43	13	45	166	220	71
5	27	33	37	15	42	184	255	113
6	25	33	48	17	64	198	275	138
7	29	36	49	20	102	217	330	167
	Glucose injected, mg. per kilo per hour							
	124	113	128	84	258	436	660	540

TABLE 5

EFFECT OF FASTING ON GLUCOSE OXIDATION IN EVISCERATED RABBITS

	*Mg. glucose/kg./hour**	
	"utilized"†	*oxidized*
Insulin—no fast	660	199
Insulin—1 day fast	540	114
Insulin—4 days fast	258	52
No insulin—4 days fast	112	30

* Av. eight-hour period.
† Glucose required to keep the blood sugar normal.

Effect of Fasting on Insulin in Glucose Oxidation. The effect of fasting in reducing the glucose "utilization" (glucose required to keep the blood sugar normal) of the eviscerated rabbit is well known.[3, 9] The fasting effect on insulin action may be studied with much greater certainty through its effect on the oxidation of glucose. The data in TABLE 5 demonstrated the reduced action of insulin on glucose oxidation during fasting.

The Effect of Insulin on the Disposition of Glucose in Eviscerated Rabbits. Although there is still much that is controversial concerning the action of insulin on glucose metabolism, there is general agreement that, in the intact animal with the blood sugar maintained at a normal level, large doses of insulin will promote the storage of glucose. Workers in the field, however, do not agree as to whether storage will only take place if the liver is functioning or whether this is a process that can be handled by the extrahepatic tissues. Recent reviewers[8] state "It is clear that doses of insulin higher than the physiologically secreted amounts of this hormone act almost exclusively on the liver, where they promote glucose-retention in a remarkable way, the ratio hepatic action/peripheral action being 25." Carbon 14-labeled glucose provides an excellent tool with which to study the influence of insulin upon sugar storage by the extrahepatic tissues. Typical data is summarized in TABLE 6. As has been noted earlier, insulin markedly increases the oxidation of glucose, leading to a large increase in C14 in

TABLE 6

EFFECT OF INSULIN ON DISPOSITION OF GLUCOSE IN EVISCERATED RABBITS

	*Control mg. glucose**	*Insulin mg. glucose**
Expired CO_2	543	2144
Body CO_2	93	360
Glucose	205	265
Water soluble fraction	980	1857
Glycogen	127	704
Crude protein	172	990
Crude fat	41	446
Fatty acids	13	127
Urine	118	209
Kidneys, lung, heart	141	415

* Glucose equivalent. Average of three animals.

the expired carbon dioxide. There is a lag in the appearance of the C14 in the expired carbon dioxide under the influence of insulin in comparison with the control animals. During this time, large amounts of glucose disappear from the blood and enter the metabolic pools. The extra glucose, which disappears under insulin action and is not oxidized, is disposed of chiefly as non-glucose, water-soluble compounds with lesser amounts in glycogen, protein, and fatty acids. Except for the water-soluble fraction, these represent a storage of glucose. The water-soluble fraction may also represent glucose storage or simply glucose en route to oxidation, the increased amount resulting from the increased glucose oxidation due to insulin.

Effect of Adrenal Cortex on Glucose Oxidation in the Eviscerated Rabbit. The influence of the adrenal cortical hormones in carbohydrate metabolism in the intact animal is recognized under certain conditions, but the role played by these hormones in the absence of the liver is less understood. Experiments have been carried out in the usual way upon adrenalectomized eviscerated rabbits, eviscerated rabbits, and the latter receiving injections of adrenal cortex extract. TABLE 7 comprises the data on these experiments. The results for the expired carbon dioxide have been expressed as

the ratio of the specific activity (SA) of the injected glucose to the SA of the expired CO_2. The SA of the circulating glucose is essentially the same as that of the injected glucose and is checked by carrying out a determination of the SA of the plasma glucose at the termination of the experiment. The ratio of the SA of injected glucose and expired CO_2 indicates the relative amounts of CO_2 coming from circulating glucose and that coming from other sources.

There was no significant change in glucose oxidation observed in the adrenalectomized animals. The data obtained by the administration of large quantities of adrenal cortex extract to eviscerated animals having intact adrenals may indicate a slight increase of glucose oxidation. The changes in oxidation are indeed small, however, in comparison with that observed after insulin administration (TABLE 4). We believe the results for experiments 10, 11, and 12 are particularly significant, since these

TABLE 7

EFFECT OF ADRENALECTOMY AND OF ADRENAL CORTEX EXTRACT ADMINISTRATION ON GLUCOSE OXIDATION IN EVISCERATED RABBITS

Hrs.	Adrenalectomized			Controls				Adrenal cortex extract*				
	R—1	2	3	4	5	6	7	8	9	10	11	12
1	12	11	35	19	27	32	19	*18	*25	17	29	27
2	9	7	27	11	20	21	15	9	13	8	14	11
3	8	6	17	9	15	17	14	6	11	6	9	6
4	7	6	13	8	14	14	13	4	9	6	7	6
5	6	5	10	8	13	12	11	3	8	*6	*6	*4
6	6	5	9	7	12	11	11	3	6	5	6	4
7	6	4	8	7	11	9	10	3	6	5	6	3
8	5	4		6	11	8	9	3	6	5	5	3

* Continuous injection of Wilson's aqueous adrenal cortex extract begun. Values given are expressed as the ratio of the Specific Activity of the injected glucose to the S.A. of the expired CO_2. The lower this figure, the greater the percentage of administered glucose being oxidized.

animals may be considered to act as their own controls. The small increase in oxidation with time is observed in all experiments of this type. We can say with certainty that there is no evidence that administration of adrenal cortex extract to eviscerated rabbits inhibits to any extent the oxidation of glucose in the extrahepatic tissues. Our results suggest that the main action of adrenal cortex in carbohydrate metabolism is on the liver.

Insulin and the Volume of Distribution of Glucose. The mechanism of immediate insulin action is unknown. The most evident result is that of causing glucose to be removed from the blood and extracellular fluids. This action is measurable and follows a very definite relationship between the dose of insulin and the amount of glucose disposed of and is undoubtedly closely connected with the basic action of insulin. Since the blood itself metabolizes relatively little glucose, this effect must involve transfer to the intracellular compartment. One view is that the glucose is freely diffusible into the cell and that the action of insulin is to promote conversion of glucose within the cell to other forms by enzyme activation, hence, the

consequent drop in glucose concentration within the cell promoting increased diffusion of the substance from the blood. The concept of free diffusion of glucose into the cell is hardly valid, since the volume of rapid distribution of labeled glucose is the same as that of thiocyanate[3] and is identical with the extracellular compartment.

Another view is that insulin increases glucose transfer into the cell through its action on a chemical mechanism. Once within the cell, the glucose molecule could enter into the metabolic chain of reactions leading to its oxidation or to its storage as glycogen or fat. A portion of this glucose might accumulate within the cell as such or as simple compounds of it. Still another possibility is that the glucose is sparingly diffusible through the cell membrane and that the intracellular metabolism of it is determined by the rate at which it will enter the cell. The metabolism of glucose would then be dependent on the rate at which it enters into the cell, and insulin might bring about its effects by increasing the permeability of the cell membrane for glucose. This view has been advanced by Levine *et al.*[10]

We planned experiments to test the influence of insulin upon the distribution of glucose by determining the volume of distribution of glucose in the eviscerated insulinized rabbit by injecting a known amount of tagged glucose and determining the degree by which this is diluted by the glucose of the body. A second method of measuring this volume is to determine how much the radioactive glucose is diluted by the body fluids. In order to reduce the correction occasioned by the transformation of tagged glucose during the mixing time for it, the plasma glucose was raised to a high level. The disappearance rate was then determined at that level by measuring the rate at which glucose had to be injected to keep the level constant. After 2 or 3 hours, when this had been ascertained, a sample of blood was taken for a plasma glucose determination and a known amount of radioactive glucose was injected intravenously. After allowing 20 minutes for mixing, a sample of blood was taken and determinations on this plasma were carried out for glucose, specific activity of the glucose, and amount of radioactivity per ml. The blood sugar was then allowed to drop until the level had dropped to a definitely lower but normal value, and the observations were repeated. The volume of distribution of the glucose was calculated by the two procedures previously discussed.[3] The results set down in TABLE 8 indicate that the volume of distribution of glucose in eviscerated rabbits is not increased by insulin. If the amount of intracellular glucose is increased by insulin, this cannot be diffusible out of the cells. If insulin increases the permeability of the cell for glucose, there cannot be any increase in the amount of this compound intracellularly as a result of insulin action. Glucose diffusion into the cell would have to be changed into some other form almost immediately on entrance.

The view of Levine *et al.*,[10] that insulin influences the permeability of cells to glucose, has been supported chiefly by the fact that, when a given dose of galactose which is not metabolized by the eviscerated nephrectomized animal is injected into such an animal, the resulting galactose concentration in the blood was much lower after insulin administration than before, indicating that some of it had been transferred intracellularly. Since galac-

tose is closely related to glucose, these workers believe that insulin might act similarly on glucose.

We have examined this matter further by the use of radioactive sorbitol, which is closely related to glucose but which is not metabolized by the eviscerated animal. Carbon 14-labeled sorbitol was administered to nephrectomized eviscerated rabbits and its dilution by the body fluids determined before and after insulin administration. Our results, in TABLE 9, show

TABLE 8
THE INFLUENCE OF INSULIN ON THE GLUCOSE SPACE OF THE EVISCERATED RABBIT

Preparation	*No.*	*Method of calculation**	
		#1	*#2*
Evisceration	1	30	30
	2	23	23
	3	22	26
Evisceration plus nephrectomy	4	25	27
	5	26	30
	6	28	28
Evisceration plus nephrectomy plus insulin	7	26	28
	8	30	
	9		23

* Results are expressed as percentage of body weight.

TABLE 9
EFFECT OF INSULIN ON THE DISTRIBUTION OF C14-LABELED SORBITOL IN NEPHRECTOMIZED, EVISCERATED RABBITS

Exp. no.	*Time in min.*			
	30	*60*	*90*	*120*
1	27*	20	20	
2	27	28	35	33
3	18	22	23	24
4	24	24	25	26

Sorbitol was injected at 0 time and insulin at 61 minutes.
* Distribution figures are expressed as a per cent of body weight.

that, under these conditions, insulin does not alter the volume of distribution of sorbitol. Sorbitol has the same structure as glucose, except for the grouping about the number one carbon. The number one carbon of galactose and glucose are alike. Insulin apparently enables galactose to enter the cell at an increased rate, where it remains as such, while glucose, which passes into the cell at an increased rate after insulin, does not accumulate, due to its metabolic alteration. The fact that sorbitol does not enter the cell with or without insulin action, suggests that entrance into the cell is dependent on a type of specificity guided by an enzyme mechanism rather than a physical process like permeability.

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THE EFFECT OF THE HYPERGLYCEMIC FACTOR AND EPINEPHRINE ON ENZYME SYSTEMS OF LIVER AND MUSCLE

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1. Metabolic Effects of Epinephrine in Intact Animals. (*a*) *Glycogenolysis.* When a small amount of epinephrine is injected into man or experimental animals, there occurs a rapid breakdown of glycogen in liver and muscle, resulting in a rise of blood sugar and blood lactic acid, the end products of glycogen breakdown in liver and muscle, respectively. The blood sugar and blood lactic acid rise very rapidly, as may be seen in FIGURE 1, where an increase was noted within three minutes after the intravenous injection of epinephrine.[9]

Several investigators[19, 1] have shown that the inorganic phosphate of blood plasma was lowered during epinephrine action, and that the urinary excretion of inorganic phosphate decreased. At the same time, hexose phosphate accumulated in muscle, and this increase was large enough to account for the fall in plasma phosphate.[10] One apparent discrepancy was noted when fasted animals with initially low liver glycogen were studied. Here, it was shown that the liver glycogen might actually increase in amount following epinephrine injection. This was explained by the Coris in 1928,[5] who found that the increased breakdown of muscle glycogen following epinephrine furnished relatively large amounts of lactic acid to the blood stream. The lactic acid was carried to the liver and was reconverted to glucose and glycogen.

It should be pointed out that these metabolic changes can be produced by very small amounts of epinephrine. In fact, they can be elicited by amounts of epinephrine which do not cause a rise of blood pressure.[9] Several other sympathomimetic amines also cause increased glycogenolysis. A recent quantitative comparison[17] of their effect on blood sugar has shown that l(−)-epinephrine was eight times more active than l(−)-norepinephrine, which in turn was much more active than d(+)-norepinephrine.

(*b*) *Other Metabolic Effects.* The oxygen consumption of the intact animal increases following the injection of epinephrine. It has been suggested[11] that the increased oxygen consumption may be related to the mobilization and subsequent metabolism of the lactic acid freed from muscle, *i.e.*, that at least part of the increased oxygen consumption may be the indirect result of glycogenolysis. This suggestion is supported by the fact that increased oxygen consumption is not noted in isolated tissues such as the diaphragm, even though increased glycogen breakdown can be demonstrated under the same conditions.

Decreased utilization of glucose after epinephrine injection has been noted by a number of investigators. The Coris, in 1928, showed clearly that peripheral glucose utilization was decreased in rats following epinephrine injection.[6] Determination of the arterio-venous blood sugar difference in

rabbits,[7] dogs,[12] and man[8, 21] during epinephrine hyperglycemia as compared to alimentary hyperglycemia has also shown a diminished uptake of glucose in muscle. Recently, it has been demonstrated that epinephrine causes a discharge of the pituitary-adrenal cortex system.[18] Since these hormones oppose the peripheral utilization of glucose, we should expect this mechanism to play some role in the decreased utilization of glucose seen in the intact animal. This view has received support from the work of Cohen.[3] A direct effect of epinephrine on the utilization of glucose by the rat diaphragm has been reported by the Walaases.[29] They noted a decrease of about

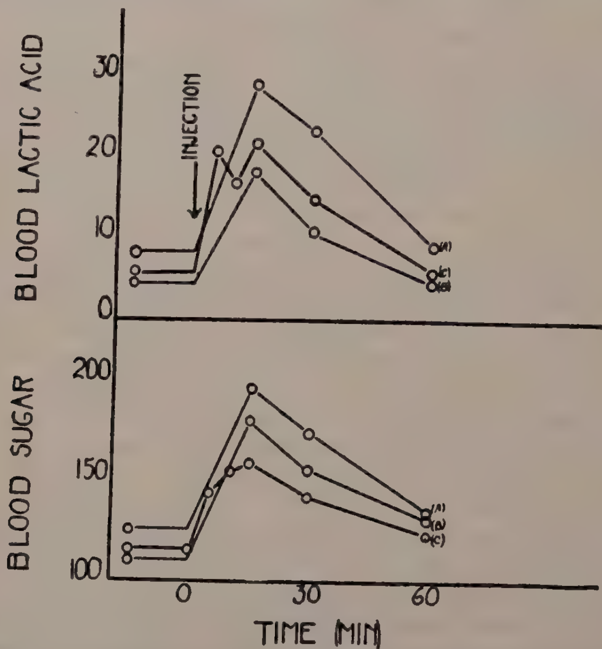

FIGURE 1. Effect of a sudden intravenous injection of 0.03 mg. of epinephrine per kilo on blood sugar and lactic acid of normal rabbits. (Reprinted from CORI, C. F., G. T. CORI, & K. W. BUCHWALD. 1930. Am. J. Physiol. 93: 273.)

30 per cent in glucose utilization when epinephrine was added to rat diaphragms incubated in glucose-containing media.

2. *The Hyperglycemic Factor of the Pancreas.* (*a*) *Evidence for Hormonal Nature.* The hyperglycemic factor is a protein which originates in the pancreas and, to a lesser extent, in the gastrointestinal tract. A large variety of other tissues did not contain the H-G factor in detectable amounts. The concentration of the H-G factor is related to the amount of islet tissue present in the pancreas. The tail or splenic portion of the dog pancreas is relatively rich in islet tissue and has much more H-G factor than the head of the pancreas. The factor is present in large amounts in the fetal calf pancreas at a stage when the digestive enzymes have not appeared and the amount of islet tissue is relatively large. Sclerosed pancreatic tissue, following duct ligation, contains an increased amount of factor per unit weight,

as may be seen in FIGURE 2, where the amount of H-G factor was assayed by the liver slice method.[23] This increase after duct ligation is in contrast to the decrease in the concentration of trypsin inhibitor, a component formed in the exocrine tissue of the pancreas. None of the H-G factor could be demonstrated in the external secretion of the pancreas even under conditions where added H-G factor could be recovered from the external secretion. Since no H-G factor could be found in liver, and since this is the major known site of action, the factor appears to have a site of origin distinct from the site of action.

The concentration of the H-G factor in the pancreas is comparable to

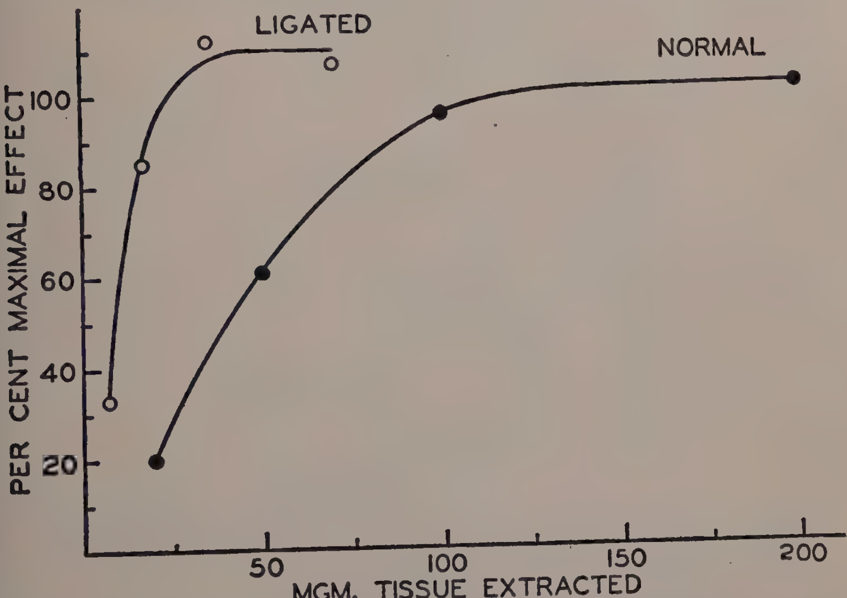

FIGURE 2. Glycogenolitic effect of extracts of the tail of normal and ligated pancreas on liver slices in per cent of maximal effect obtained by addition of "insulin." (Reprinted from SUTHERLAND, E. W. & C DE DUVE. 1948. J. Biol. Chem. 175: 663.)

that of insulin in terms of biological response. FIGURE 3 shows the blood sugar changes following the intravenous injection of pancreatic extracts into rabbits. An amount of normal pancreas which contains enough insulin to produce hypoglycemia, in general contains enough hyperglycemic factor to produce hyperglycemia. Extracts from the pancreas of alloxan diabetic animals contain normal amounts of H-G factors, while insulin is absent (FIGURE 3). This points to the alpha cells as the probable site of formation of the H-G factor.

The purified hyperglycemic factor is effective in very small amounts.[2, 24] Microgram quantities cause glycogenolysis in liver slices and hyperglycemia in intact animals. When added to cell free systems, the H-G factor has no apparent enzymatic activity *per se* and has no effect on the enzymatic breakdown of glycogen in these systems. Its action seems to depend on the control mechanisms of the intact cells.

(*b*) *Action in the Intact Animal.* When a small amount of the hyperglycemic factor is injected intravenously, there follows a rapid rise of blood sugar, as may be seen in FIGURE 4.[25] The rapidity of the effect is similar to that seen with epinephrine. With the factor, however, there is no rise of blood lactate. This indicates that a single injection of the factor does not influence the breakdown of muscle glycogen. Experiments by Collens and Murlin published in 1929[4] indicated that the site of the hyperglycemic effect was in the liver. Injection of small amounts of an "insulin" preparation into the portal vein of a dog gave rise to a hyperglycemia, whereas the

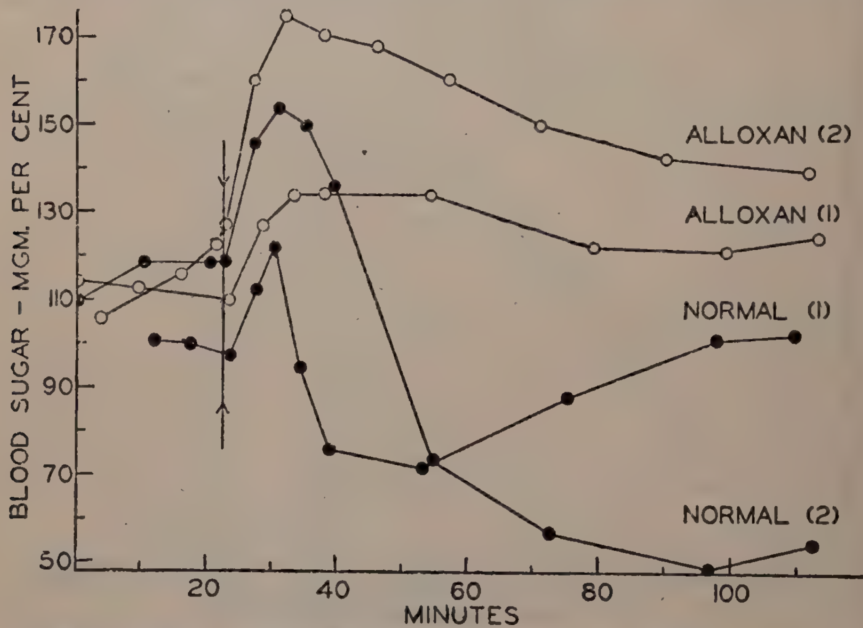

FIGURE 3. Blood sugar changes following intravenous injection of pancreatic extracts of normal and alloxan diabetic rabbits into normal rabbits. Curves 1 resulted from the injection of an extract representing approximately 0.5 gm. of pancreas; Curves 2 from 1.5 gm. of pancreas. (Reprinted from SUTHERLAND, E. W. & C. DE DUVE. 1948. J. Biol. Chem. **175**: 663.)

same amount injected into the jugular vein gave no hyperglycemic response. Other possible biological effects of the H-G factor have not been adequately studied.

3. *Action of the H-G Factor and Epinephrine on Liver Slices.* An assay system based on the glycogenolytic effect of H-G factor on liver slices has been described.[22–24] The H-G factor and epinephrine increase glycogen breakdown and glucose output in liver slices, and it is possible to obtain graded responses to increasing concentrations of these agents. Half maximal stimulation with l(−)-epinephrine occurred at a concentration of about one part per 15 million in a test system which consisted of 1.2 cc. of a phosphate saline buffer containing one liver slice of about 70 mg. weight (FIGURE 5).[26] l(−)-Norepinephrine and d(+)-epinephrine were about $\frac{1}{6}$ as active, while d(+)-norepinephrine was much less active. Ephedrine had a weak

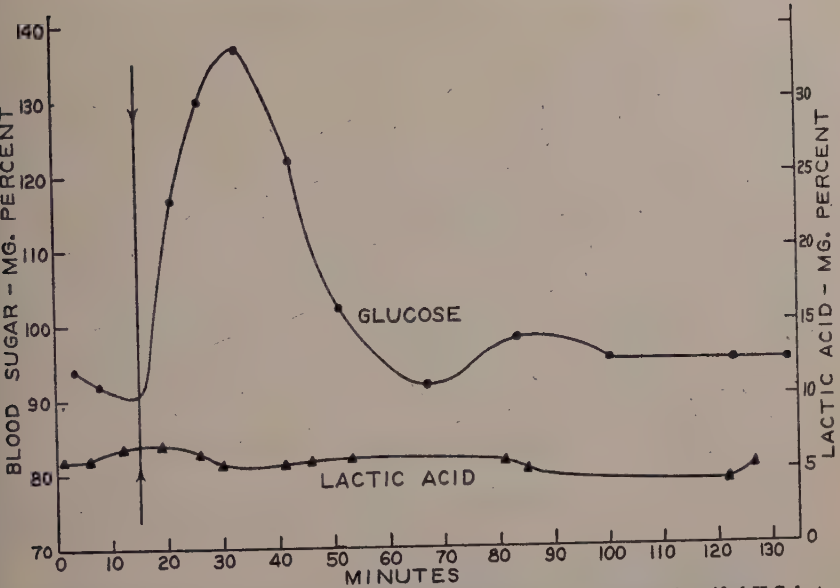

FIGURE 4. Blood sugar and lactic acid changes following intravenous injection of purified H-G factor into a rabbit. (Reprinted from Proceedings of the Laurentian Hormone Conference, New York, 5: 441, 1950.)

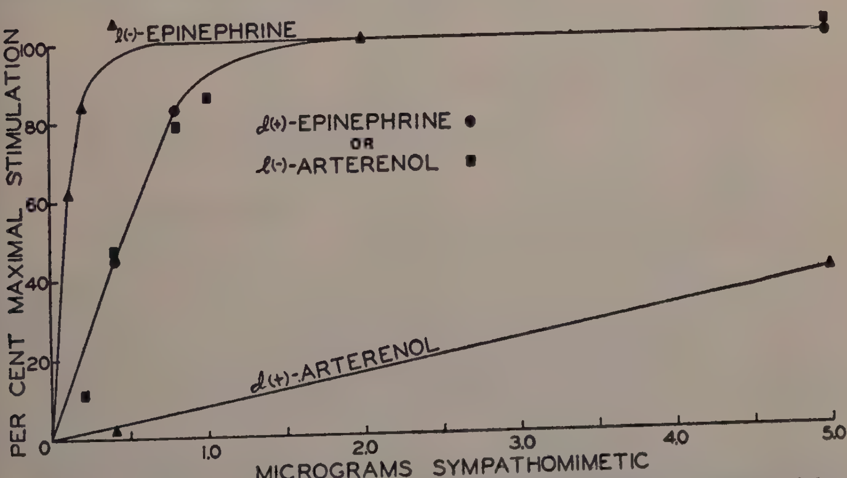

FIGURE 5. Stimulation of glucose output of rabbit liver slices by sympathomimetics. Slices were shaken in 1.2 ml. of 0.02 M phosphate-saline buffer pH 7. Control slices were incubated without addition of a sympathomimetic. After 40 minutes of shaking at 37°, barium hydroxide and zinc sulfate were added and the filtrate was analyzed for glucose. Each point is an average of two experiments. (Reprinted from SUTHERLAND, E. W. & C. F. CORI. 1951. J. Biol. Chem. 188: 531.)

and variable activity, while benzedrine was inactive. These activities in the liver slice test system parallel the order of hyperglycemic activity in the intact animal.

A maximal increase of glycogen breakdown was reached on addition of about 2 μg. of purified factor or 0.3 μg. of epinephrine in the usual test

system. The same maximum was reached with either agent, and the H-G factor and epinephrine were not additive in their effect when each was added in supramaximal amounts. Under a variety of experimental conditions, the glycogenolytic effect of the two agents was influenced in the same manner. Their action appeared to be on the same enzyme in liver.[25]

Three enzymatic reactions are involved in the conversion of glycogen to glucose in the liver. Their sequence is shown in FIGURE 6. It was of interest to determine which of the three was the slow or rate-limiting reaction, because it seemed probable that the action of the glycogenolytic agents would be on that step. It was found that the phosphorylase reaction was the rate-limiting step in liver slices and in liver homogenates. When either glucose-1-phosphate or glucose-6-phosphate was added to liver slices, the rate of glucose formation was greatly increased (FIGURE 7). This shows that mutase plus phosphatase can dispose of much more glucose-1-phosphate

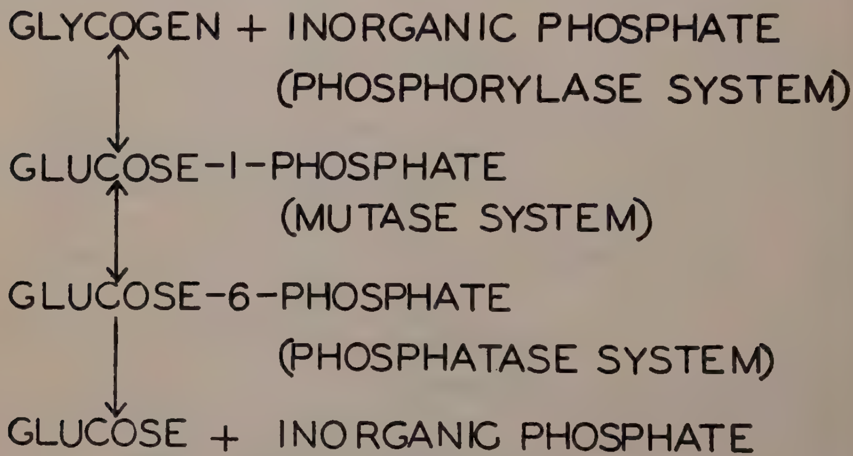

FIGURE 6. An outline of the conversion of glycogen to glucose by the liver.

than is formed by phosphorylase in slices (or homogenates) with glycogen and phosphate being present in optimal amounts. When liver homogenates were studied, it was possible to show that the addition of crystalline phosphoglucomutase did not significantly increase the rate of formation of glucose, while the addition of crystalline phosphorylase greatly increased the rate of glucose formation.

Since the phosphorylase reaction appeared to be the slow step, it seemed desirable to investigate the effect of concentration of inorganic phosphate, which is a component in this reaction. It was found that the effect of the glycogenolytic agents was increased when inorganic phosphate was added. This phosphate effect was obviously related to the phosphorylase reaction. The effect of H-G factor and epinephrine on the permeability of slices to phosphate was next studied. It was found by chemical analyses and by use of radioactive inorganic phosphate that the permeability of the slices to inorganic phosphate was not altered by the H-G factor and epinephrine.

While the preceding results showed that the phosphorylase reaction

limited the rate of glycogen breakdown, they did not prove that the effect of the H-G factor and epinephrine was on this reaction. An increase in the activity of mutase or phosphatase could speed up the over-all reaction by removing glucose-1-phosphate at a faster rate. This would result in a lower level of glucose-1-phosphate or glucose-6-phosphate in the slices stimulated by the H-G factor or epinephrine. On the other hand, if the phosphorylase reaction were stimulated, the glucose-1-phosphate level should increase. To settle this point, it was necessary to measure the concentration

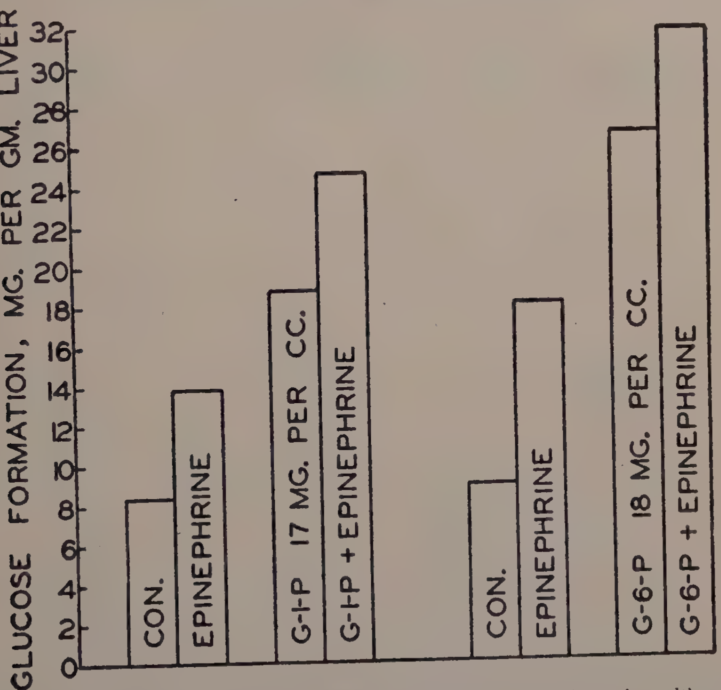

FIGURE 7. Effect of addition of glucose-1-phosphate (17 mg. per ml., as the dipotassium salt) and of glucose-6-phosphate (18 mg. per ml.) on the glucose output of liver slices, with and without the addition of epinephrine. The period of incubation was 45 minutes at 37°. (Reprinted from SUTHERLAND, E. W. & C. F. CORI. 1951. J. Biol. Chem. **188:** 531.)

of glucose-1-phosphate in the slices. This was done by means of the isotope technique (FIGURE 8). The concentration of glucose-1-phosphate (and glucose-6-phosphate) was distinctly higher in the slices incubated with H-G factor or epinephrine than in the control slices. These findings led to the conclusion that both the H-G factor and epinephrine stimulate the phosphorylase system in liver slices.

4. Effect on Phosphorylase Content of Liver Slices. Further investigation of the phosphorylase reaction has shown that the concentration of active enzyme is increased by the H-G factor or epinephrine. This has been demonstrated directly by measurements of phosphorylase activity in homogenates or extracts prepared from slices preincubated without or with these agents.[26] FIGURE 9 summarizes two experiments which illustrate

this point. The time of preincubation is written on the bars: C refers to control slices; E to slices incubated with epinephrine; and F to slices incubated with the hyperglycemic factor. The slices at zero time contained all of the phosphorylase in its active form. This is usually the case when no special precautions are taken in the killing of the animal and the removal of the liver. The first experiment shows the loss of phosphorylase activity when slices were incubated for thirty minutes at 37°. The homogenates

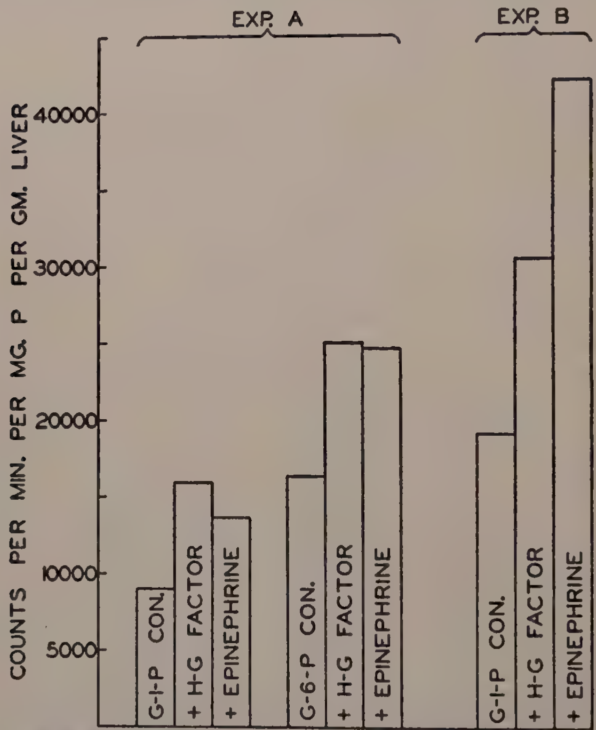

FIGURE 8. Concentration of glucose-1-phosphate and glucose-6-phosphate in liver slices incubated without and with the addition of glycogenolytic agents. Isotopic hexose phosphates were formed by incubation with radioactive inorganic phosphate and were isolated by the carrier technique described in the text. Samples of P^{32} with different specific activities were used in Experiments A and B. (Reprinted from SUTHERLAND, E. W. & C. F. CORI. 1951. J. Biol. Chem. **188**: 531.)

from slices incubated with epinephrine or the H-G factor had considerably more phosphorylase activity than the control. The second experiment shows that epinephrine can restore phosphorylase activity very rapidly once it has decreased. After 20 minutes of incubation at 37°, the phosphorylase activity of the control had fallen considerably, and there was only a slight additional decrease of phosphorylase during the next twenty minutes. After twenty minutes of incubation, epinephrine was added to some of the slices. Within four minutes, the phosphorylase activity had almost returned to the original value, and the effect was as large after four minutes of incubation as after fifteen minutes of incubation with epinephrine. The same results were obtained when the hyperglycemic factor was used

in place of epinephrine. It should be pointed out that this increase in the phosphorylase content of liver slices can also be demonstrated when phosphorylase is tested in the direction of synthesis, *i.e.*, formation of polysaccharide from glucose-1-phosphate in homogenates prepared from slices. Actually, routine testing is usually carried out in the direction of synthesis at pH 6 in the presence of fluoride to minimize any action of the mutase system on glucose-1-phosphate.

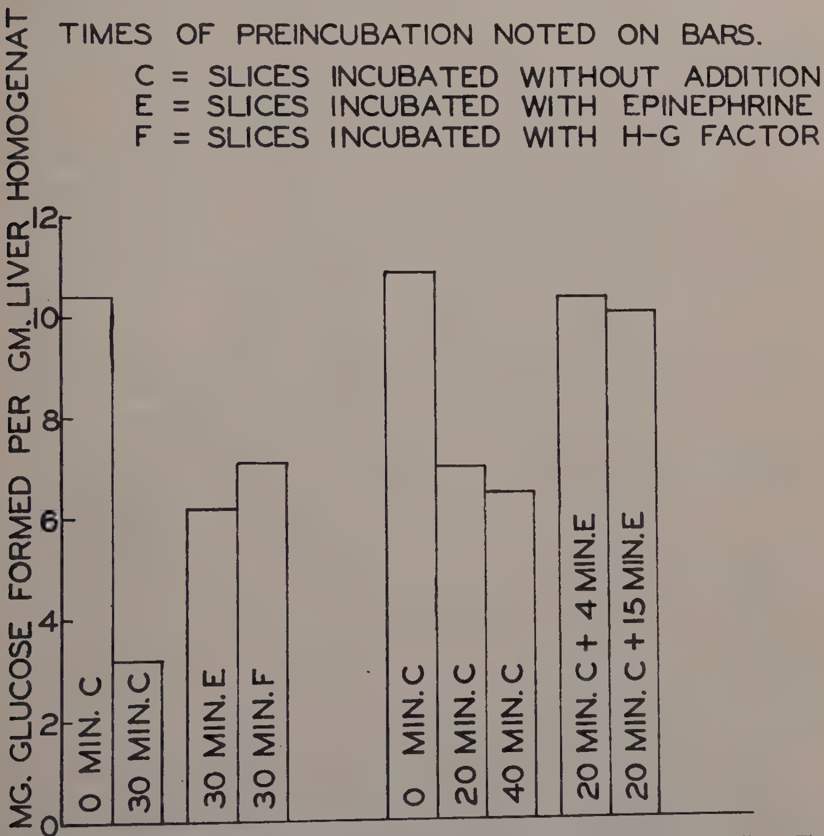

FIGURE 9. Phosphorylase activity in homogenates prepared from preincubated rabbit liver slices. The rate of glucose formation was measured at pH 7.4 in 0.04 M phosphate buffer and 1.0 per cent glycogen.

So far, the resynthesis of active phosphorylase has been observed only in the intact tissue. When the cell structure is destroyed, by freezing and thawing of liver slices or by homogenization, addition of epinephrine or H-G factor no longer causes an increase in the amount of active phosphorylase. Fluoride likewise does not cause resynthesis of active phosphorylase under these conditions, while it has such an effect when added to liver slices (see below).

An increase in the phosphorylase content of the liver has also been observed in intact animals after injection of epinephrine. The changes were smaller than in liver slices, because it is difficult to remove samples of liver

from an intact animal without some stimulation of phosphorylase activity through the animal's own epinephrine secretion.

One possible interpretation of the experiments presented in this section is that the level of active phosphorylase in the liver represents a balance between inactivation and resynthesis of the active form of phosphorylase. This is supported by the following additional observations. Fluoride in 0.1 molar concentration is an effective inhibitor of the phosphorylase-inactivating enzyme which can be extracted from liver, muscle, and other tissues. When fluoride is added to liver slices, it prevents the fall in phosphorylase which occurs during incubation at 37°, and it also causes a resynthesis of active phosphorylase when it is added after the phosphorylase content has decreased. Except for the 100,000-fold difference in the effective concentration, fluoride is, in this respect, similar to epinephrine and the H-G factor. Fluoride differs, however, from these two agents in that it inhibits the inactivation of phosphorylase in liver homogenates, while the two glycogenolytic agents are without effect. This indicates that fluoride shifts the above balance in favor of active phosphorylase by preventing inactivation. Epinephrine and H-G factor would then act by accelerating the resynthesis of active phosphorylase.

5. Effect of Epinephrine on Muscle Phosphorylase. Lesser demonstrated in 1920[16] that the addition of epinephrine to isolated frog muscle caused an increase in the rate of glycogen breakdown. Hegnauer and Cori[15] showed that the increased glycogen breakdown in frog muscle incubated anaerobically with epinephrine was accompanied by an accumulation of hexose phosphates and a corresponding decrease of inorganic phosphate. Riesser studied the effect of epinephrine on isolated rat diaphragms and found that epinephrine caused glycogen breakdown even under conditions where the diaphragm would be synthesizing glycogen if epinephrine were absent.[20] The Walaases[29] made a carbohydrate balance on diaphragms incubated in a glucose-containing medium and found that addition of 2 micrograms of epinephrine caused glycogen breakdown, increased lactic acid formation, and accumulation of hexose-phosphate esters. Here, again, the direction of phosphorylase activity was reversed.

Phosphorylase has been isolated from muscle in two forms. Phosphorylase *a* is obtained from muscle of deeply anesthetized animals and is active without addition of adenylic acid. Phosphorylase *b* is formed *in vivo* when muscle is stimulated to fatigue, or *in vitro* when phosphorylase *a* is incubated with the PR enzyme of muscle.[13] The *b* form is inactive unless adenylic acid is added. A reversibility of the $b \rightarrow a$ transformation has been observed in the intact animal when previously stimulated muscle was allowed to recover.[14] Recently, we have been able to demonstrate that epinephrine increases the amount of active phosphorylase in muscle; *i.e.*, of phosphorylase *a*, which is active in the absence of adenylic acid.[28] This is shown in FIGURE 10, where rat diaphragms were incubated at 37° in phosphate-saline buffer. The amount of phosphorylase *a* (shaded portion of bar) decreased rapidly, while the total phosphorylase activity (the whole bar), measured in the presence of adenylic acid, remained unchanged. In

experiment A, the decrease in phosphorylase *a* was largely prevented when epinephrine was present in the medium from the start of incubation. Experiment B shows that, when epinephrine is added after 20 minutes of incubation of the diaphragm, there occurs a restoration of phosphorylase *a* activity within 3 minutes.

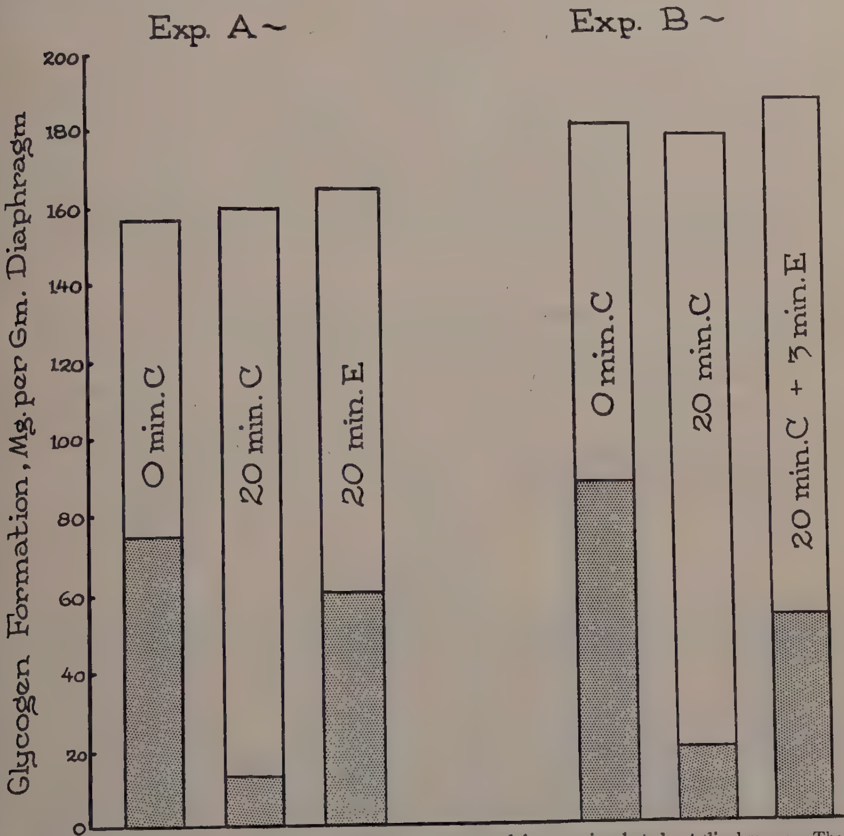

FIGURE 10. Phosphorylase activity in homogenates prepared from preincubated rat diaphragms. The times of preincubation are noted on the bars, C = diaphragms incubated without addition, E = diaphragms incubated with epinephrine. 20 min C + 3 min E means that the diaphragms were first incubated for 20 minutes without addition, followed by 3 minutes of incubation in the presence of epinephrine. The rate of glycogen synthesis from glucose-1-phosphate was measured at pH 6.1 in the presence of 0.05 M NaF. The results are expressed in mg. of glycogen per gm. diaphragm for a period of incubation of 15 minutes at 37°. The total height of the bar represents the phosphorylase activity with adenylic acid. The height of the shaded area represents the activity without adenylic acid.

These results duplicate those with liver slices, except that the inactive form of liver phosphorylase does not correspond to the *b* form of muscle, since it is not activated by adenylic acid. This difference requires further investigation.

6. *Enzymatic Inactivation of Phosphorylases.* Most tissues (muscle, liver, spleen, pituitary, aorta) contain enzymes which inactivate muscle and liver phosphorylase *in vitro*. In the case of muscle, the inactive form is the *b* form, which can be detected by an activity test in the presence of adenylic acid. Liver phosphorylase has recently been purified.[27] It

differs in solubility and other physical characteristics from muscle phosphorylase *a*, and the inactive form is different from the *b* form of muscle, since it does not show activity or is only partially reactivated in the presence of adenylic acid.

The enzymatic inactivation of phosphorylase presents many interesting aspects, which are being investigated at the present time. Table 1 lists some of the compounds which were tested for their effect on the inactivating enzyme of liver when acting on liver phosphorylase.[28] The same results are obtained when inactivating enzymes from other tissues are used. Muscle adenylic acid, inosinic acid, and, to a lesser extent, ribose-5-phosphate, in the concentrations indicated, exert a strong inhibitory effect on the enzymatic inactivation of liver or muscle phosphorylase. Fluoride in 0.1 M concentration likewise protects the two phosphorylases. Yeast adenylic acid, adenosine, ribose, glucose-6-phosphate, and the pyrimidines are without effect on the rate of enzymatic inactivation. The significant fact, that epinephrine and the H-G factor do not owe their effect to an inhibitory action, has already been mentioned. The purines listed in the third column have a strong accelerating effect on the enzymatic inactivation of liver phosphorylase and a small effect on that of muscle phosphorylase. The most active of this group were the methylated xanthines, which doubled the rate of inactivation of liver phosphorylase in a concentration of 2×10^{-4} molar. Higher concentrations caused a 4- to 5-fold increase in the rate of inactivation.

Table 1

Effect of Various Compounds on the Rate of Enzymatic Inactivation of Liver Phosphorylase*

Inhibitory†	*No effect*	*Acceleratory*
Adenosine-5-phosphate 1×10^{-4} M	Adenosine-3-phosphate	Adenine 1×10^{-3} M
Inosine-5-phosphate 1×10^{-4} M	Adenosine	Hypoxanthine
Ribose-5-phosphate 2×10^{-3} M	Uridine	Xanthine
Fluoride 1×10^{-1} M	Cytosine	Theobromine
	Thymine	Theophylline
	Ribose	Caffeine 2×10^{-4} M
	Glucose-6-phosphate	
	Epinephrine	
	H-G factor	

* Purified liver phosphorylase was incubated with the inactivating enzyme from liver.

† Also inhibitory when the test system consists of crystalline muscle phosphorylase *a* and purified PR enzyme of muscle.

It is of interest that the accelerating effect of adenine is lost when either ribose or ribose-3-phosphate is attached to position 9 of the purine ring, and that substitution of ribose-5-phosphate converts adenine into a compound which is inhibitory.

Summary. The amount of active phosphorylase in liver and muscle represents a balance between inactivation and resynthesis of the active form. The change from the inactive to the active form can be extremely rapid, so that the active enzyme in liver or muscle may be doubled within a few minutes. The balance within the intact cell is under hormonal con-

trol, as shown by the fact that minute quantities of epinephrine can cause a large increase of active phosphorylase in liver slices and in rat diaphragms. The hyperglycemic factor of the pancreas acts on liver slices in the same manner as epinephrine. An increasing amount of evidence indicates that this hyperglycemic factor is a hormone.

Most tissues contain enzymes which inactivate liver and muscle phosphorylase *in vitro*. Muscle adenylic and inosinic acid and, to a lesser extent, ribose-5-phosphate exert a strong inhibitory effect on the inactivating enzymes. Fluoride in 0.1 M concentration is likewise inhibitory. Yeast adenylic acid and adenosine were not inhibitory. A number of purines, including the methylated xanthines, had a marked accelerating effect on the enzymatic inactivation of liver phosphorylase.

The resynthesis of active phosphorylase has been observed so far only in intact liver and muscle tissue. The effect of epinephrine is contrasted with that of fluoride. Although both agents cause resynthesis of active phosphorylase in liver slices, the former does not inhibit enzymatic inactivation of phosphorylase, while fluoride does. This suggests that epinephrine influences the balance between active and inactive phosphorylase by promoting synthesis, while fluoride acts by preventing inactivation.

There is evidence that, when epinephrine is injected into intact animals or added to isolated diaphragms incubated in a glucose medium, the direction of phosphorylase activity may be reversed, so that it proceeds mainly in the direction of glycogen breakdown. This problem requires further investigation.

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EFFECTS OF CORTISONE AND PURIFIED PITUITARY GROWTH HORMONE ON KETOGENESIS BY SURVIVING LIVER SLICES*

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Since Burn and Ling[1] first described the ketonuric effect of crude pituitary extracts twenty-one years ago, many investigators have confirmed the fact that injected pituitary extracts produce ketonemia and ketonuria.[2, 3] After it had been indicated by the work of Collip and his colleagues[4] and by the studies of Mirsky[5] that the liver is essential for the ketonemic response, the effects of crude pituitary extracts on the metabolism of surviving liver slices were studied by Shipley,[6] by Ennor,[7] and by Campbell and Davidson.[8] While there are certain points on which these authors do not agree, their collective work stands in support of the view that the liver is a site of action of the ketogenic substance or group of substances that can be extracted from the pituitary gland. Shipley[6] was the only one of these investigators who described enhancement of ketogenesis by surviving liver slices when crude pituitary extract was added to the system *in vitro*. This constituted the only available evidence that a ketogenically active material in crude extracts causes the liver to produce ketone bodies at an accelerated rate by stimulating the liver cells directly, and not merely by offering them an excess of lipid precursor mobilized from the fat depots.

When purified pituitary hormones became available for physiologic study, Bennett and coworkers[9] described enhanced ketonemia in response to both growth hormone and ACTH. In fact, their data indicate a pronounced synergistic effect on the level of ketonemia between these two substances.

When viewed in the light of the studies cited above, the report of Bondy and Wilhelmi[10] appeared to contain a certain paradox. These authors, in the course of well-designed and carefully executed experiments, found a ketogenic defect in liver slices obtained from rats many months after hypophysectomy and described their efforts to repair this defect by injecting certain hormones. They found that, while ketogenesis by liver slices of hypophysectomized rats could be restored to essentially normal levels by thyroxin administration, the injection of purified growth hormone *in vivo* was without effect. These results were clearly difficult to reconcile with those of earlier workers, who used crude extracts or purified hormones in intact animals. The fact that the rate of ketogenesis by the slices could be accelerated by thyroxin administration[10] did not appear to us to rule out the possibility that lack of other hormones may have contributed to the ketogenic defect in the hypophysectomized rat. Accordingly, the experi-

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† With the technical assistance of Janet M. DeWitt.

ments to be described were begun, initially with the purpose of repeating certain parts of Bondy's and Wilhelmi's study.

Materials and Methods. Male hypophysectomized rats were obtained from the Hormone Assay Laboratory in Chicago. Intact rats of the same strain (Sprague-Dawley) were received with each shipment of hypophysectomized animals. All of these rats, which were used in the *in vivo* injection experiments, were maintained on a diet of Red Heart canned dog food. The intact rats, which were used for the *in vitro* hormone experiments, were maintained on a diet of Purina Dog Chow. All rats received water *ad libitum.*

The authors are greatly indebted to Mr. Irby Bunding and the Armour Company for generous supplies of purified pituitary Growth Hormone and crystalline bovine plasma albumin and for characterizations of these materials. Growth Hormone preparations 22KR1 and J-21609R were prepared according to the method devised by Wilhelmi, Fishman, and Russell.[11] The former preparation is approximately equal in growth hormone potency to the Armour Standard, 22KR2, and contains 5 per cent or less of Thyroid Stimulating Hormone (expressed as per cent of the activity of a standard preparation of TSH) as its only known major contaminant. Preparation J-21609R is rated at 80 per cent of the activity of 22KR2 standard, with approximately 10 per cent of the activity of a standard TSH preparation as its only known major contaminant. It is of interest to note that some of the well-known growth hormone studies of deBodo[12] and Young[13] on carbohydrate metabolism and of Levin[14] on fat mobilization were done with 22KR1.

Ketone bodies were determined as acetone according to the method of Greenberg and Lester[15] on barium sulfate filtrates of the medium in which liver slices were incubated. Since only negligible amounts of ketone bodies were extracted from representative slices of both treated and untreated livers at the time they were prepared for incubation, initial values were not determined routinely. Ketones are expressed as acetone per 100 mgs. wet liver. This convention was adopted because fat and water content analyses of representative liver samples from all of the groups showed no significant differences when any pair of groups was compared. Since no studies were made of the linearity of the rate of ketone body production, the amount produced in the entire run (2 hours) is given.

Measurements were made of oxygen consumption by the direct method. Approximately 100 mg. of liver slice (0.3 mm. thick) were suspended in 2.8 ml. of a Krebs Henseleit[16] phosphate buffer of pH 7.4 in standard Warburg flasks of approximately 13.5 ml. capacity. The center well contained 0.2 ml. of 20 per cent KOH, and all runs were made in oxygen. No substrate was added to the system in any of the experiments to be reported. Q_{O_2} values were calculated on the basis of representative dry weight rather than final dry weight.

Experimental Design and Results. (*a*) *Injection of Hormones* in Vivo. The experimental results will be given more or less in the order in which they were obtained. For the sake of clarity, some of the data will be repeated from table to table as the need arises for making new comparisons.

It was of interest to determine how soon following hypophysectomy the ketogenic defect of surviving liver slices becomes apparent. In this connection, it was possible to study four rats which had been hypophysectomized for less than 15 days. All of the rats were fasted overnight. Livers were removed and sliced three hours after an intraperitoneal injection of physiological saline. Ketone body production by the slices was measured over a two-hour period.

TABLE 1 gives a comparison between the performance of slices obtained from these animals and those of both intact rats and rats hypophysectomized for 30 days or longer. Rats studied in the immediate post-operative period yielded tissues which were intermediate in performance between those of intact animals and those of rats long after hypophysectomy. It should be

TABLE 1

EFFECT OF HYPOPHYSECTOMY ON KETOGENESIS BY SURVIVING RAT LIVER SLICES

Group	*No. of rats*	*No. of vessels*	*Ketones as acetone mcg./100 mg. wet/2 hrs.*	*S. E.*
A Intact controls	4	20	126	±3.4
B Hypox < 15 days	4	22	105	±2.5
C Hypox > 30 days	8	45	87	±2.1

Probabilities: AB < 0.01; BC < 0.01.

TABLE 2

EFFECT OF GROWTH HORMONE INJECTED *in Vivo* ON KETOGENESIS BY LIVER SLICES OBTAINED FROM RATS SOON AFTER HYPOPHYSECTOMY

Group	*No. of rats*	*No. of vessels*	*Ketones as acetone mcg./100 mg. wet/2 hrs.*	*S. E.*
B Hypox < 15 days	4	22	105	±2.5
D Hypox < 15 days plus GH, 3 mgs	4	22	129	±4.0

Probability: < 0.01.

noted here that, since it was not feasible to check the completeness of hypophysectomy histologically, some incompletely hypophysectomized rats may have been included in the "early" group. This was less likely in the case of the "late" group, since it was possible to reject a few rats which gained more than 20 gms. in weight.

It was possible to study four rats given Growth Hormone 22KR1 during the immediate post-operative period. These rats were sacrificed three hours after having received a single intraperitoneal injection of 3 mgs. of the growth hormone preparation.

TABLE 2 shows that this treatment restored the rate of ketogenesis of the surviving liver slices of these animals to a normal level.

Later, working with rats 30 days and more after operation, the treatment with growth hormone described above was found to be completely without effect, as can be clearly see in TABLE 3. These results are in good accord

with the negative results reported by Bondy and Wilhelmi,[13] who worked with a growth hormone preparation which was made by the process adopted for the isolation of 22KR1.

The observation that injection of a GH preparation has a positive effect within 15 days of hypophysectomy assumed some importance in elucidating a mechanism of the failure of the material to influence hepatic ketogenesis long after operation in both Bondy's experiments and our own. It is well known that, in the two weeks following hypophysectomy, there is only an incomplete atrophy of those target organs which are stimulated by trophic hypophysial hormones. Prominent among these, of course, is the adrenal cortex, which had been implicated in the problem of the ketogenic response to crude pituitary extracts by the work of Fry[17] and that of MacKay.[18]

The question was asked: does the growth hormone require the presence of some adrenal cortical activity in order to stimulate ketogenesis in the liver? It is now widely recognized that many hormonal effects cannot be elicited in the absence of a certain minimal amount of adrenal cortical hormone or hormones. Therefore, it was necessary to test the effect of GH in cortisone pre-treated hypophysectomized rats long after operation. An experiment on the effect of the cortisone-priming procedure itself was also indicated.

TABLE 3

LACK OF EFFECT OF GROWTH HORMONE INJECTED *in Vivo* ON KETOGENESIS BY LIVER SLICES OF HYPOPHYSECTOMIZED RATS 30 DAYS AND MORE POST-OPERATIVELY

Group	*No. of rats*	*No. of vessels*	*Ketones as acetone mcg./100 mg. wet/2 hrs.*	*S. E.*
C Hypox > 30 days........	8	45	87	±2.1
E Hypox > 30 days plus GH, 3 mgs............	6	31	93	±2.4

P > 0.10.

Accordingly, nine "late" hypophysectomized rats were injected with 1 mg. of cortisone acetate (Cortone, Merck) intramuscularly at 5 P.M. Following an overnight fast, they were given an additional mg. of the same material at 8 A.M. Three hours later, the animals were sacrificed and liver slices were incubated for the usual two-hour period. The results of this experiment are given in TABLE 4. There is a small, but statistically significant, increase in rate of ketogenesis over that of slices obtained from appropriate hypophysectomized controls. It is possible that a more valid estimate of this difference could have been presented if determinations of liver glycogen had been made. While there appears to be an effect attributable to the cortisone in this experiment, it can hardly be represented as a restoration of ketogenic activity to normal levels.

Five similar rats were treated with cortisone in the manner described above. In addition, each was given an intraperitoneal injection of 3 mgs. of GH 22KR1 at 8 A.M. and sacrificed three hours later. Slices obtained from these rats produced ketone bodies at a rate that was far above that

seen in the cortisone group and not significantly different from the value for slices obtained from intact rats (TABLE 4).

It is of some interest that the effect of growth hormone injection on the

TABLE 4

EFFECT OF CORTISONE ALONE AND IN COMBINATION WITH GROWTH HORMONE ON KETOGENESIS BY LIVER SLICES OF HYPOPHYSECTOMIZED RATS 30 DAYS AND MORE POST-OPERATIVELY

Group	*No. of rats*	*No. of vessels*	*Ketones as acetone mcg./100 mg. wet/2 hrs.*	*S. E.*
C Hypox > 30 days........	8	45	87	±2.1
F Hypox > 30 days plus Cortisone 2 mgs.........	9	39	97	±3.9
G Hypox > 30 days plus cortisone 2 mgs. plus GH 3 mgs.............	5	24	121	±3.6

Probabilities: CF < 0.01; FG < 0.01.

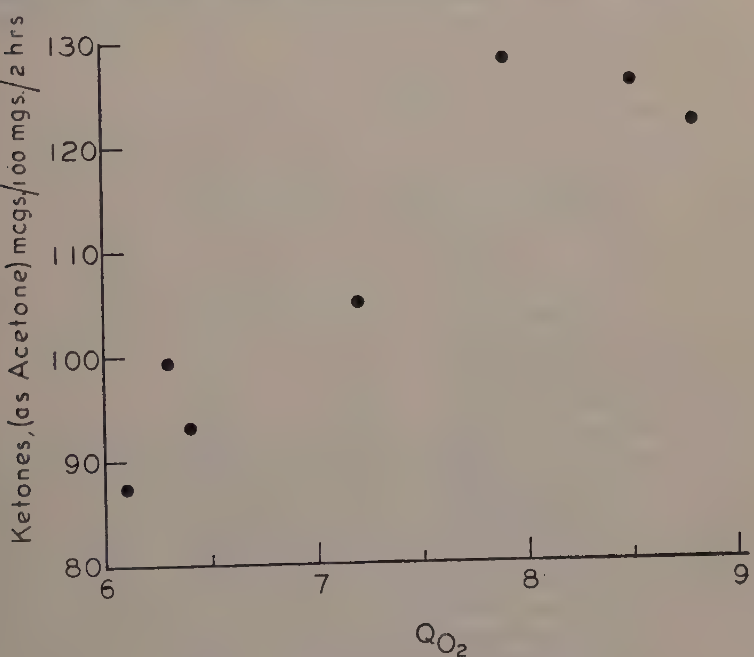

FIGURE 1. Ketogenesis and oxygen consumption.

oxygen consumption of the slices paralleled the effect on ketogenesis rather closely. This observation, shown graphically in FIGURE 1, is consistent with the findings of Campbell and Davidson.[8]

(*b*) *Effect of Growth Hormone Added* in Vitro *on Ketogenesis by Liver Slices.* The demonstration of an effect of growth hormone injected *in vivo* on ketogenesis by surviving liver slices does not constitute proof that the hormone stimulates ketogenesis by acting directly on the liver cells. Al-

though there were no demonstrable increases in liver fat content three hours after the injection of growth hormone in the experiments reported above, it is entirely possible that the increased rate of ketogenesis by the liver slices may have been a passive, secondary response to the active mobilization of depot fat by the hormone or hormone combination. The definitive proof of a direct action of growth hormone on the liver cells requires that an effect be demonstrated when the liver slice is confronted with the hormone *in vitro*.

It will be recalled that Shipley[6] had described enhancement of ketone body production by normal liver slices incubated in the presence of rat serum and crude saline pituitary extract. On the other hand, Campbell and Davidson[8] were unable to obtain such an effect with the pituitary extract they used. Bondy and Wilhelmi[10] state that purified growth hormone had no effect on ketone body production when incubated with liver slices from normal or hypophysectomized animals.

For this series of experiments, adult, intact male rats were used as sources of liver slices. The rats were not fasted, because it was believed that there was a greater chance of showing stimulation of ketogenesis under conditions in which the control rates were kept as low as possible. Eight slices were prepared from the liver of each rat: four were placed in vessels which contained 2.8 ml. of phosphate-buffered Krebs Henseleit medium and 0.2 ml. of KOH; the other four were incubated in buffer which contained 1 mg. of growth hormone per ml. of buffer, or a total of 2.8 mgs. per flask. The flasks were oxygenated and shaken at 112 oscillations per minute for two hours. The raw data of the first series of experiments, done with 22KR1, are shown graphically in FIGURE 2. It is evident that the liver slices incubated with growth hormone 22KR1 made significantly more ketone bodies than did control slices. Even if one ignores variance and compares the mean of all of the control vessels with that of all of the hormone vessels, the probability that this result could be due to chance is far less than 0.01 according to student's "t" test.[19]

If one expresses these data as per cent increase over the control value, using the means of the quadruplicate determinations in each case, the results are as shown in FIGURE 3. In addition, the results of four similar experiments with preparation J-21609R are shown.

Six experiments were done in which Growth Hormone J 21609R was denatured by heating in a boiling water bath for 30 minutes. It is evident that there is no consistent change in ketone body production by slices incubated with the denatured hormone. In retrospect, however, the heat denatured hormone experiment did not appear to be an adequate control for the growth hormone experiments, because heating precipitated the protein, while the growth hormones were highly soluble in the concentrations used. It seemed possible that the small effect we had seen might have been a non-specific one, and that it could be reproduced by incubating the slices with an indifferent, soluble protein in the same concentration. Crystalline beef plasma albumin (Armour) was selected as such an indifferent protein, and it was possible to do 4 experiments with this substance. In-

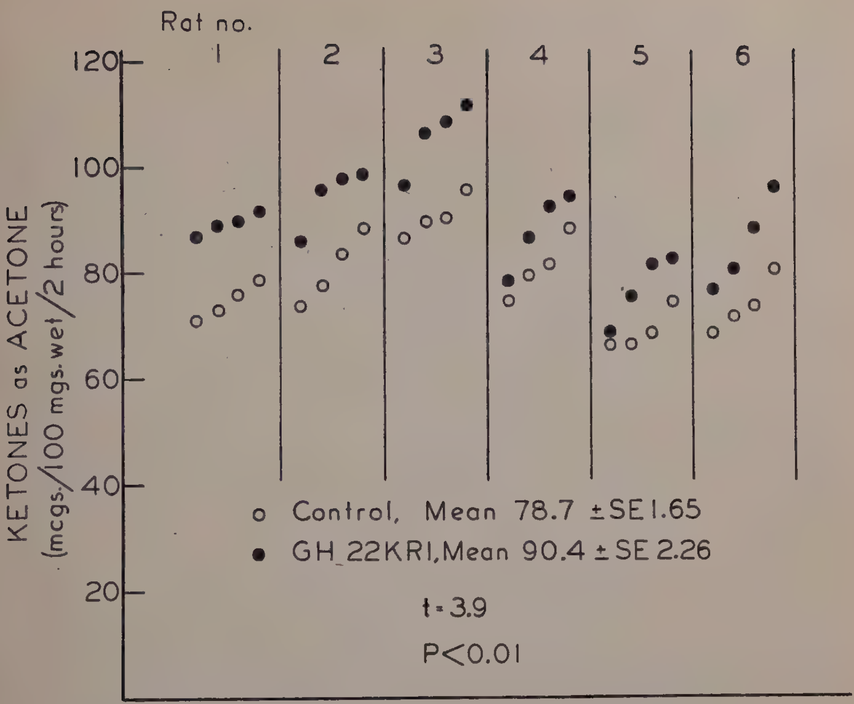

FIGURE 2. Effect of growth hormone added *in vitro* on ketogenesis by surviving liver slices.

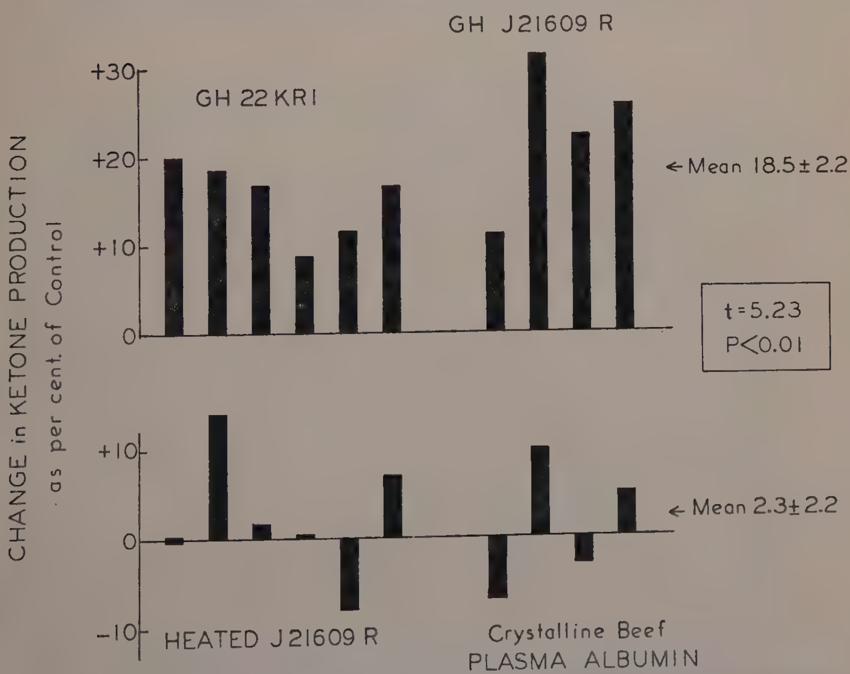

FIGURE 3. The effect of the addition of growth hormone *in vitro* on ketogenesis by liver slices of fed, intact rats.

spection of FIGURE 3 clearly reveals that 3 mg. per ml. of crystalline albumin has no consistent effect on the rate of ketogenesis by surviving liver slices.

Conclusions. Liver slices obtained from hypophysectomized rats make significantly fewer ketone bodies than do slices obtained from intact rats. Injection of a purified pituitary growth hormone preparation into rats within 15 days post-hypophysectomy effectively repairs the ketogenic defect of their liver slices. Thirty days, and more, postoperatively, the injection of growth hormone alone is without effect on ketogenesis by liver slices. Cortisone injection resulted in a small but statistically significant rise in rate of ketone body formation. When hypophysectomized rats were pretreated with cortisone and then injected with growth hormone 50 days and more postoperatively, ketogenesis by their liver slices was at the normal rate. The incubation of liver slices of intact rats with two preparations of growth hormone added *in vitro* resulted in a statistically significant increase in ketone body production. Denatured growth hormone and crystalline bovine plasma albumin in the same concentration were without effect.

Addendum. Since this paper was written, it has been found that preparation J-21609 R (Armour) consistently causes an increase in rate of ketogenesis when added *in vitro* to liver slices obtained from 350–400 gm. rats, but not in those obtained from 200 gm. animals. Further, when J-21609 R and two other preparations of comparable growth hormone potency (kindly supplied by Dr. Alfred Wilhelmi) were tested simultaneously on slices from the same rat, the Armour material stimulated ketogenesis while the other two did not. The results of these experiments will be reported in detail elsewhere.

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IN VITRO INFLUENCES OF CORTICOSTEROIDS ON PHOSPHORYLATING ENZYMES

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In the search for the mechanism by which the adrenal cortical hormones influence metabolism, it was originally supposed, on the basis of analogy, that certain phosphorylating enzymes might be involved (1939). Since 1940, attempts have been made to study the problem *in vitro*.

In the following lines, it is not intended to relate our findings to those on the living animal, and space does not permit a discussion of the problem in relation to others. We shall confine ourselves to the description of the experiments of our own laboratory on isolated tissues *in vitro*.

Glycogen Phosphorylase. In a series of papers between 1940–1945, we studied the phosphorylase action in minced muscle. If glycogen is added to minced muscle ("Brei") in a phosphate buffer solution, inorganic phosphate is bound to glycogen and glucose-1-phosphate is formed. The further breakdown has to be inhibited by the addition of NaF. The decrease of inorganic P in the solution is estimated after 7, 15, 30, and 60 minutes at 20°C. Lohmann and Meyerhof first studied this reaction, and Schuman also made a few experiments on adrenalectomized animal muscle. The kinetics of the reaction were described by Montigel (1943).

Verzár and Montigel (1941) showed that glycogen phosphorylation *in vitro* is diminished in the muscles of adrenalectomized rats (1941), cats, and dogs (1943). They then showed (1942) that the decreased phosphorylation can be restored with desoxycorticosterone (FIGURE 1). The experiments were confirmed in our laboratory by Doetsch (1944) and by Staehelin and Voegtli (1948).

Montigel (1945) studied 53 adrenalectomized rats, eight adrenalectomized cats, and two adrenalectomized dogs. TABLE 1 shows our rat experiments.

The activity of progesterone *in vitro* was found to be about 20 times less than that of DOC (desoxycorticosterone). Testosterone and oestradiol had no significant action. FIGURE 1 shows some curves from our publications (1941). The decrease in glycogen phosphorylation velocity has been found also with liver-Brei in our laboratory by Doetsch (1944) and by Stähelin and Voegtli (1948) (TABLES 2 and 3).

Besides corticosteroids and progesterone, we found that glutathion and also cysteine have an activating action on the decreased phosphorylation of adrenalectomized rat muscle. This is in agreement with the observation of Williams and Watson (1940) on kidney and on bone-phosphatase, and the observations of Cori and coworkers (1945) on pure phosphorylase enzyme.

FIGURE 2 shows phosphorylation of glycogen in the muscle of the hind leg in four normal cats and in adrenalectomized cats. These were first kept alive and healthy with daily doses of DOCA (desoxycorticosteroneacetate). If this was left out and the signs of adynamia appeared, the velocity of

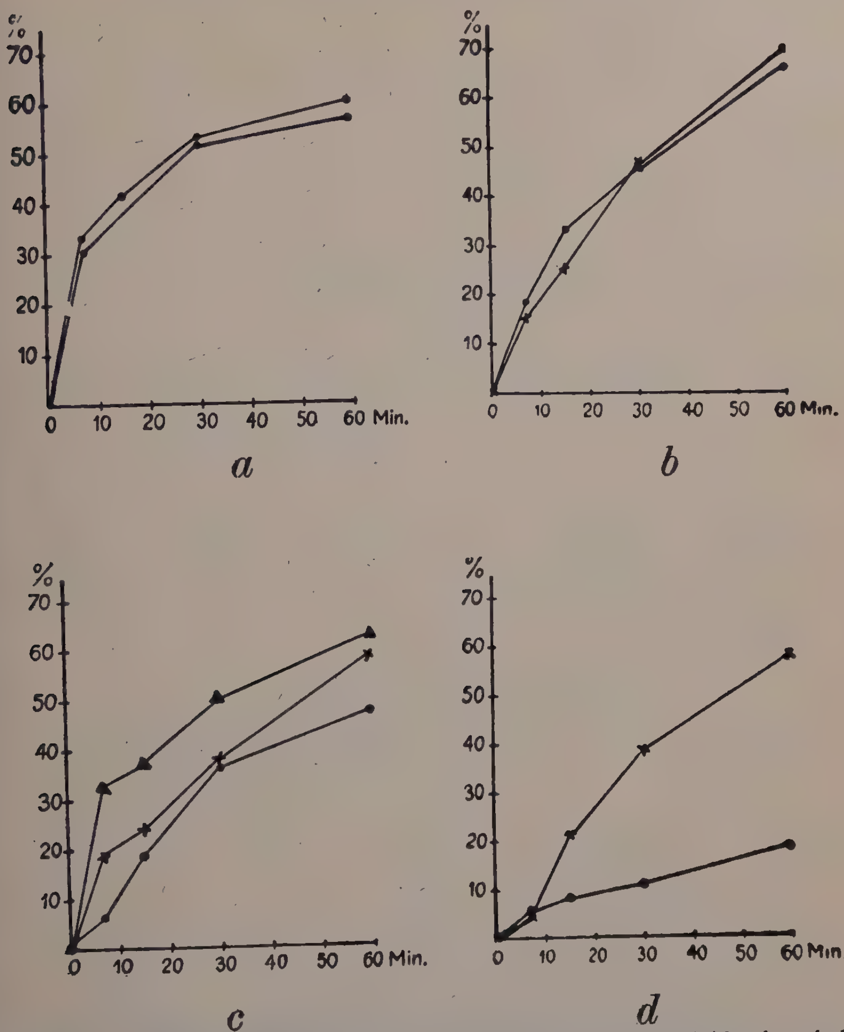

FIGURE 1. Glycogen phosphorylation with minced rat muscle. (a) Normal, 20°C. (b) Adrenalectomized, compensated with DOCA treatment *in vivo*. (c) Adrenalectomized rat: ● without DOC; × with 0.1% DOC; ▲ with 1% DOC *in vitro*. (d) Adrenalectomized rat ●; same with 1% DOC *in vitro* ×.

TABLE 1

GLYCOGEN PHOSPHORYLATION WITH MINCED MUSCLE*

	Number of animals	*Addition*	*mg. % P decrease after*	
			15 min.	*30 min.*
Normal............	10	—	52.6	67.6
Adrenalectomized....	45	—	33.5	50.1
Adrenalectomized....	30	0.1% DOC	46.3	62.8
Adrenalectomized....	9	2% progesterone	48.1	59.7

* After Montigel, 1945.

glycogen phosphorylation decreased (curves 5, 6, and 7). Three other adrenalectomized cats were kept healthy with daily injections of DOCA and showed the perfectly normal other three curves (8, 9, and 10) of FIGURE 2.

In two of these animals (7 and 8), pieces of the anterior extensor muscles of the hind leg were extirpated during adynamia, in ether narcosis. The animals were then cured with large doses of DOCA and the curves 7b and 8b were observed with pieces of muscle cut out from the same parts of the other leg a few days later.

The decrease of glycogen-phosphorylation was criticized by Helve and by Riesser, whose experiments were negative. In the later papers of Montigel, of Doetsch, and of Stähelin and Voegtli, however, we confirmed our original observations of a decrease. The restoration of decreased phosphorylation with corticosteroids is still being studied. It was found in several experimental series, but it was not observed in others. How far the presence of cysteine and other factors are influencing it, we do not know as yet, but we are convinced that we have not found all the factors in such a reaction. We have, therefore, also abstained from speaking of corticosteroids as anything else than a "velocity factor" of glycogen phosphorylation, while others have used the word of "coferment," presumably too early.

TABLE 2

GLYCOGEN PHOSPHORYLATION WITH MUSCLE AND LIVER*

Organ		Number of animals	mg. % P decrease after	
			15 min.	30 min.
Muscle	normal	32	41.6	59.4
	adrenalectomized	57	26.4	44.1
Liver	normal	18	26.2	33.8
	adrenalectomized	16	7.8	15.0

* After Doetsch, 1945.

TABLE 3

GLYCOGEN PHOSPHORYLATION WITH MUSCLE AND LIVER OF RATS*

Organ		Number of animals	mg. % P decrease after		
			7 min.	15 min.	30 min.
Muscle	normal	20	24	45	65
	adrenalectomized	65	16	31	52
Liver	normal	39	10	22	35
	adrenalectomized	24	8	12	24

*After Staehelin and Voegtli, 1947.

Alkaline Phosphatase of the Absorption Epithelium in the Intestinal Mucosa and the Kidney Tubules. The first assumption of a phosphorylation disturbance in the intestinal epithelium after adrenalectomy was based on analogies (Verzár and McDougall, 1936). Then Kutscher and Wüst (1942) confirmed, directly, a disturbance of the alkaline phosphatase of the intesti-

nal mucosa of adrenalectomized guinea pigs. Verne (1948) has shown, with the histological method of Gomori (1941), that the alkaline phosphatase of the intestinal mucosa, which lies in high concentration in the lumen adjacent part of the epithelial cells, is greatly diminished after adrenalectomy and is restored with adrenal cortical extracts.

Our own experiences with rats will be published in detail with Sailer

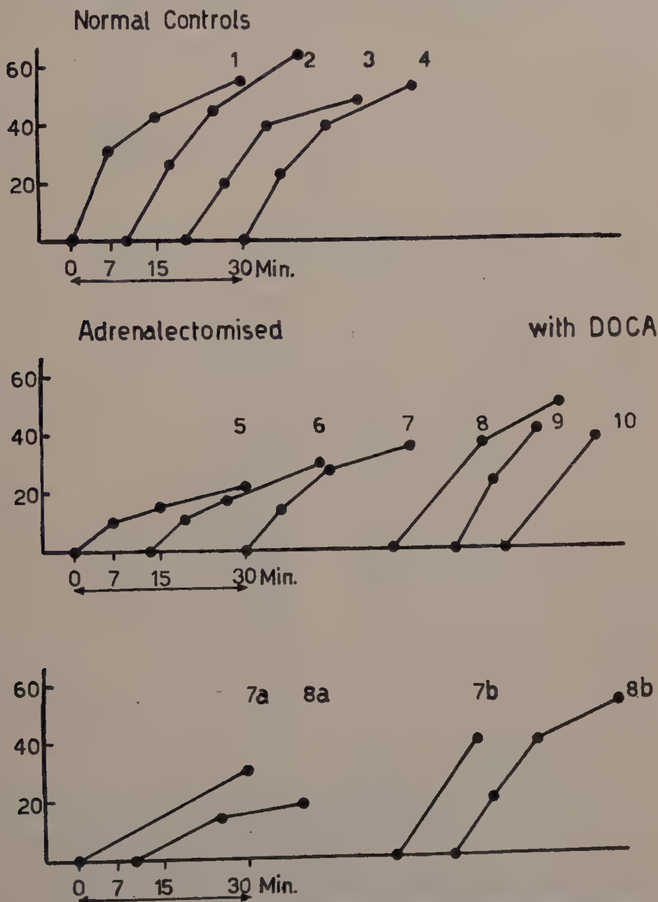

FIGURE 2. Glycogen phosphorylation by muscle (see text for description).

(1951), but the following summary may be given. The mucosa was scratched away from the upper half of the small intestine. The alkaline phosphatase activity of 20 cm. mucosa was measured with glycerophosphate at pH 9.2 *in vitro*. The increase of inorganic phosphate was estimated colorimetrically. The activity decreased with the time after adrenalectomy, more or less parallel with the decrease of body weight and the development of an adynamic state, as FIGURE 3 shows.

We succeeded in bringing the value of alkaline phosphatase activity of the intestinal mucosa to normal with DOCA, Cortisone, or adrenal cortical

extract if these were injected in the animal *in vivo,* at least 12–24 hours in advance. We have not succeeded as yet, however, in restoring the activity *in vitro* also.

Similar results have been shown after adrenalectomy on the alkaline phosphatase of the kidney by Kutscher and Wüst (1942), with chemical methods, and by Tissières (1948), in our laboratory, with the histological

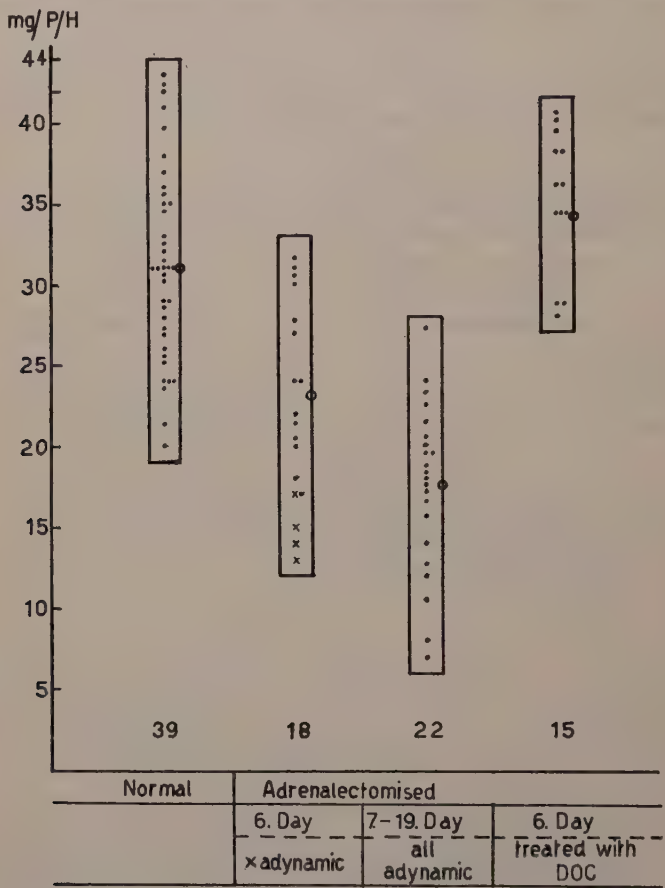

FIGURE 3. Alkaline phosphatase of upper small intestine in rats: ▬ mean values; ● single experiments.

method of Gomori. The epithelial cells of the kidney tubules have a reabsorption activity, similar to those of the intestinal epithelium. Their alkaline phosphatase disappears after adrenalectomy.

In Vitro *Glycogen Production of Surviving Muscle.* After Gemmil (1940) had found that the surviving diaphragm of the rat produces glycogen *in vitro* in considerable quantities, we studied (Verzár and Wenner, 1947, 1948) the influence of steroid hormones, especially corticosteroids, on this process.

The surviving diaphragm was kept at 38°C. in oxygenated buffered

Ringer's solution. Glucose and insulin were added and an increase of 100 to 200 per cent of the original glycogen quantities was seen after 60 or 90 minutes.

These experiments were done on resting diaphragm and on working diaphragm. For this purpose, each diaphragm was cut in three equal parts. One gave the original glycogen content, the second the glycogen production in rest, and the third, glycogen production after rhythmical stimulation for one hour. TABLE 4 is a summary of a large number of rat experiments by Verzár, Mentha, and Voegtli (1948). The work of the diaphragm was measured in arbitrary units and can be used only in a comparative way without great accuracy. Glycogen production was estimated in Ringer's solution (38°C and oxygenated) with or without 100 mg. or with 400 mg.

TABLE 4

GLYCOGENESIS, GLYCOGENOLYSIS, AND GLYCOGEN CONSUMPTION IN REST AND WORK OF RAT DIAPHRAGM

Solution	*Normal*					*Adrenalectomized*				
	Glycogen mg. %			*work cm2*	*No. of exp.*	*Glycogen mg. %*			*work cm2*	*No. of exp.*
	change		*con-sumed during work*			*change*		*con-sumed during work*		
	rest	*work*				*rest*	*work*			
Ringer solution	− 85	−245	*160*	27	25	− 40	−145	*105*	22	10
With glucose 100 mg.%	+ 35	−145	*180*	32	23	+ 45	− 50	*95*	36	8
With glucose 400 mg.%	+ 80	−110	*190*	41	10	+ 60	− 35	*95*	28	5
With glucose 100 mg.% + 1 U insulin	+170	− 90	*260*	36	11	+ 90	− 40	*130*	32	6
With glucose 400 mg.% + 1 U insulin	+365	+ 10	*355*	38	16	+290	+ 80	*210*	28	11

glucose per 100 cc. and also with the addition of 1 U insulin per 100 cc. Experiments were made with normal and with adrenalectomized rats (sixth day after adrenalectomy). The glycogen consumption is calculated as the difference between glycogen in the resting and the working muscle. Glycogen consumption was much higher if, besides glucose, insulin was also present, and especially high with the relatively very high glucose concentration of 400 mg. per cent. All values have been calculated for p and t values and are significant (see the original papers).

The adrenalectomized animal diaphragm has shown in every series decreased values for glycogen production and glycogen consumption. In general, the mechanical work production was somewhat smaller (not very considerably, however). TABLE 4 shows this.

The activity of desoxycorticosteroneacetate on the glycogen metabolism of normal and adrenalectomized animal diaphragm was rather unexpected. With Wenner (1947), we found an almost total inhibition of glycogen for-

mation if, besides 400 mg. per cent glucose and insulin (1 to 100 U per 100 cc.), DOC was added in quantities of 5 mg. per cent. These experiments were repeated with Mentha and Voegtli (1948) and extended to the working muscle, of both normal and adrenalectomized animals. Large series of animals were used and all results were calculated for probability, their p and t values.

TABLE 5 shows that glycogen production in 100 or 400 mg. per cent glucose with insulin is smaller in the adrenalectomized animal diaphragm. However, considerable quantities are produced. Similarly, the glycogen used (or produced) during muscle work is less in the adrenalectomized animal. The influence of the addition of 5 mg. per 100 cc. of DOC is very clear in both groups. The glycogen consumption and glycogen production are inhibited.

With 100 mg. glucose and 1 U insulin per 100 cc., the glycogen production in rest is almost totally inhibited, as is the (calculated) glycogen consumption during work, by about 50 per cent. Interestingly enough, the mechanical work did not decrease to any measurable degree.

The results were the same with adrenalectomized animals. All values are smaller, however, than in the similar series with normal muscle.

These experiments thus show: (1) that, *in vitro*, there is a diminished glycogen metabolism of the muscle during rest and work after adrenalectomy; and (2) that desoxycorticosterone has an intensive inhibitory activity on glycogen production and breakdown, in both normal and adrenalectomized animal muscle.

These results were confirmed in 1949 (Bozovic, Leupin, and Verzár) on the anterior abdominal muscles of the mouse, which are as thin as the diaphragm and can therefore be used in the same way. Insulin and glucose greatly increase the glycogen production, while DOC completely inhibits it. It was shown that adrenaline also gives an inhibition of glycogen production. Adrenaline and DOC together gave an augmented effect. It seemed that the inhibition of glycogen production has to be explained as an increase of glycogen breakdown.

In addition to desoxycorticosterone, compound E was also tested. It, too, has an inhibitory effect on glycogen production *in vitro*. With anterior abdominal muscle of the mouse, we could show also that the glucose which disappears from the solution is only partly (about ½) used for glycogen production. This was confirmed by Bartlett and MacKay (1949) about the same time.

Other Organs. We did not succeed, however, in getting significant glycogen production with liver cuts of rats *in vitro* either from glucose or from pyruvate, with various modifications of Ringer's solution. This has to be mentioned in view of experiments of Chiu and Needham (1950) who, in some cases, saw somewhat increased glycogen production under the influence of corticosteroids. Large series worked out at our laboratory by Leupin (unpublished) showed no significant differences.

If we now try to find an explanation for the inhibition of glycogen production in the muscle by corticosteroids and to relate it to other facts of the action of corticosteroids *in vitro*, we might repeat that it was shown in the

TABLE 5
INFLUENCE OF DESOXYCORTICOSTERONE (5 MG. PER CENT) ON GLYCOGEN CONSUMPTION OF ISOLATED DIAPHRAGM

Solution	*Normal*								*Adrenalectomized*							
	Glycogen mg. %						*work cm2*		*Glycogen mg. %*						*work cm2*	
	change				*consumption during work*				*change*				*consumption during work*			
	rest		*work*						*rest*		*work*					
		DOC		*DOC*		*DOC*		*DOC*		*DOC*		*DOC*		*DOC*		*DOC*
Ringer..............................	−85	−45	−245	−140	*160*	*95*	27	26	−40	−40	−145	−117	*105*	*77*	22	39?
With glucose 100 mg. % + 1 U insulin	+170	+15	−90	−115	*260*	*130*	36	34	+90	+20	−40	−60	*130*	*80*	32	34
With glucose 400 mg. % + 1 U insulin	+365	+165	+10	−25	*355*	*190*	38	35	+290	+65	+80	−10	*210*	*75*	28	30

first part of this paper that, in adrenalectomized animals, glycogen phosphorylation by phosphorylase was diminished. This is the first phase of glycogen breakdown. Corticosteroids restored it. Thus, if we find, with isolated diaphragm or abdominal muscle *in vitro*, that DOC increases glycogen breakdown and inhibits glycogen production, we have actually done the same as with muscle-Brei. With DOC, we increased the activity of "phosphorylase," the enzyme acting in the breakdown of glycogen in the muscle.

One may doubt whether this is a physiological action of the hormone. We have called attention to the possibility that the addition of corticosteroid to the system *in vitro* might have an action of "competitive inhibition" in an enzyme system. Besides an analogy with large intoxicating doses, however, we have not found any facts to support such a theory.

It will need more work to correlate these observations on isolated tissues with the facts observed in the living animal.

In Vitro *Reactions on Electrolyte Metabolism of Surviving Tissues*. Starting with the observations on the activity of corticosteroids in the living animal, we studied the relation of carbohydrate and electrolyte metabolism *in vitro*.

The first *in vitro* reaction in which an electrolyte, potassium exchange was shown to be coupled to glycogen metabolism was found by Pulver and Verzár (1940). Yeast cells *in vitro*, after the addition of glucose, form a polysaccharide (as Willstätter showed), and we found that potassium (K) then enters the cells. In the next period, when the carbohydrate is fermented to ethyl alcohol, the K again leaves the cells. The same reaction was also described by us with surviving leucocytes *in vitro* (Pulver and Verzár, 1940b). We have shown that K is taken up together with glucose and glycogen is formed during the first 10 minutes. Then lactic acid is produced and K leaves the cells again. Finally, we also demonstrated this mechanism with surviving muscle (diaphragm and abdominal muscle) *in vitro* (Leupin and Verzár, 1950) (FIGURE 4).

Without the addition of glucose, the glycogen content in Ringer's solution (38°C. oxygenated, 90 minutes) decreases, and K leaves the tissue and passes into the solution. If glucose is added (200 mg. per cent and 1 to 200 U insulin per 100 cc. Ringer's solution), large quantities of glycogen are produced and K either does not leave the muscle or, in many cases, is taken up by it. Here again, if the glycogen production was inhibited with desoxycorticosterone, no K uptake was found.

This connection between glycogen and potassium metabolism was confirmed by Leibowitz and Kupermintz (1942) on *Escherichia coli*. It is also known in erythrocytes (Danowski, 1941, and Harris, 1941) and brain tissue (Dixon, 1949). We have to refer to the discussions on the explanation of this phenomenon by Conway and Breen (1945) and Conway and Malley (1946), after whom it is an ionic exchange, and Muntz (1947), Boyer, Lardy, and Phillips (1942, 1943), Lardy and Ziegler (1945), Rothstein and Enns (1946), Orskov (1948) and, finally, Cowie, Roberts and Roberts (1949). The last-named authors used radioisotope K^{42} (as had Hevesy and Nielson, 1941) and discussed the formation of a fructose-1,6 di-kalium phosphate during glycogen breakdown. The problem is related to the question of how K is concentrated inside certain cells, as in muscle,

etc. We have discussed this in other places in connection with the theory of muscular contraction (1943).

Thus, on isolated tissues *in vitro*, a connection of carbohydrate with electrolyte metabolism was shown. Whatever its mechanism may be, there is no doubt that production of glycogen or its breakdown also leads to electrolyte changes. We have called attention to this fact, together with findings on the living animals, and discussed its bearing on the theory

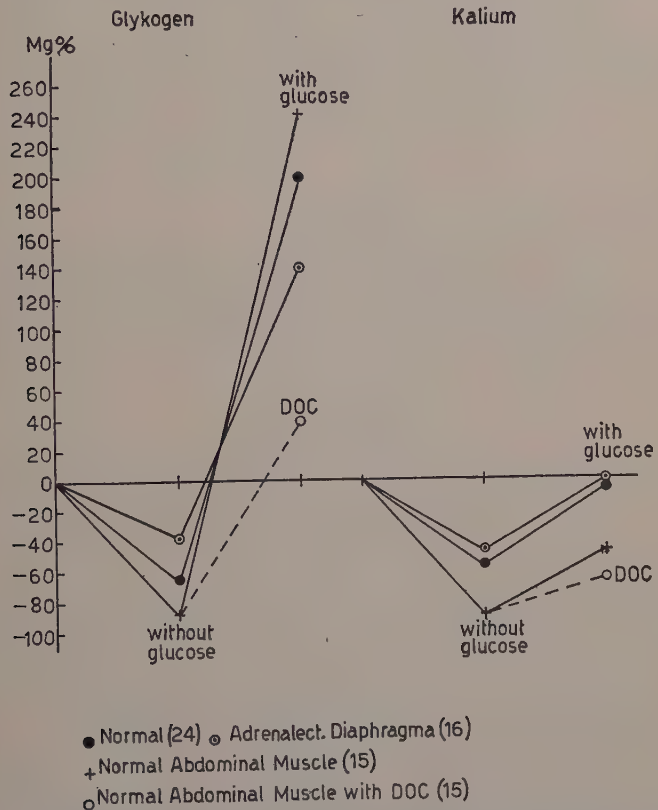

FIGURE 4. Connection of glycogen production and K metabolism of surviving muscle. Mean values; numbers of experiments in ( ); each period, 90 minutes; changes of glycogen and K in mg. per 100 g. muscle.

of the existence of differently acting "carbohydrate" and "electrolyte" hormones of the adrenal cortex (Verzár and Wang, 1949 and Wirz, 1950).

Summary. (1) The influence of adrenalectomy on phosphorylase activity was studied *in vitro* with minced muscle and liver.

(2) Glycogen phosphorylation is decreased. It can be shown with rat and cat muscle. With the latter, the decreasing phosphorylation during development of a crisis can be demonstrated.

(3) Corticosteroid treatment, also with DOCA, keeps muscle phosphorylase normal.

(4) *In vitro*, the restoration of the decreased glycogen phosphorylation was successful in several series with desoxycorticosterone, but much less

so with progesterone. An activation was also seen with glutathion and cysteine.

(5) *In vitro* surviving muscle (rat diaphragm, mouse abdominal wall muscle) produces large quantities of glycogen from glucose both without and, more so, with the addition of insulin.

(6) Glycogen production was inhibited completely by desoxycorticosterone and perhaps somewhat less (?) by cortisone. This inhibition may be an increased glycogen breakdown through activation of glycogen phosphorylase. The inhibition of glycogen metabolism by corticosteroids is present with normal and with adrenalectomized rat muscle, both with resting and with working muscle.

(7) *In vitro*, an activation of glycogen production of muscle is produced by insulin and an activation of glycogenolysis by adrenaline and by corticosteroids.

(8) Potassium metabolism is influenced *in vitro*, together with carbohydrate metabolism in yeast cells, leucocytes, and muscle. Glycogen production from glucose leads in all these objects to potassium uptake, while glycogen breakdown (fermentation) releases potassium from the cells. Different explanations and the relation of this fact to the theory of the metabolic action of corticoids are mentioned.

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